https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

DEDICATED TO

THE MEMORY OF MY FATHER

ProQuest Number: 10647053

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10647053

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

URINARY FREE THYROID HORMONES IN CHILDREN:

AN ASSAY BY GAS LIQUID CHROMATOGRAPHY

by

ABDELHAMID ABDELKADIR MANSOUR

M.B., B.Ch., D.C.H.

Thesis submitted to the University of Glasgow

for the degree of

Doctor of Philosophy

April 1978

ACKNOWLEDGEMENTS

The studies presented in this thesis were carried out by the author in the Steroid Laboratory of the University Department of Child Health, Royal Hospital for Sick Children, Glasgow. The investigation was supported by finances from the Egyptian Government.

I am especially indebted to Dr. W. Hamilton for his unfailing patience and constant encouragement and guidance. I would also like to thank Professor Hutchison and Professor Cockburn for allowing me access to their patients.

I am grateful to Mr. J. Devlin, Department of Medical Illustration for the preparation of the photographs in this thesis, and also to Miss Eleanor Greig and Miss Joan Gray for typing this volume.

The patience and unfailing confidence of my wife and family has been a great source of strength to me throughout the preparation of this work.

CONTENTS

Page

CHAPTER 3: RESULTS WITH DISCUSSION

CHAPTER I

INTRODUCTION

Historical Resumè

According to Mettler (1947), Egyptian--Ebers's papyrus (1500 BC) makes mention of thyroid enlargement as one of the conditions which requires surgical treatment.

Albucasis (10th century), one of the greatest Arabian surgeons, differentiated congenital from acquired goitre. In his opinion only the latter was suitable for surgery, and then only when it was not too large (Jantsch, 1948; Liek, 1929). The Persian physician, Sayed Ismail Al-Jurjani (circa AD 1136) postulated a connection between exothphalmos and goitre (Rolleston, 1936).

Naturally this interest in the thyroid gland stimulated attempts to discover its function and the factors causing abnormalities of that function. The relationship of the thyroid to various body functions was studied by experimental thyroidectomy (Cooper, 1827), but little information was gained as death generally followed due to the removal of the parathyroid glands as well. The concept of a substance released by the gland was formulated nine years later (King, 1836). Using a simple microscope, King noted that the thyroid was composed of follicles filled with a gummy, translucent fluid. In 1883 both Reverdin & Reverdin (1883) and Kocher (1883) noted the similarity of clinical myxoedema and the state which developed after successful removal of the thyroid. This observation led naturally to the injecting of a glycerine

extract of thyroid to relieve myxoedema (Murray, 1891) and later to the oral feeding of lightly cooked sheep thyroid to patients with this condition (Howitz, 1893). The stage was thereby set for the development of such clinical and biochemical investigation which has led to our present knowledge of thyroid function.

In 1895 Baumann isolated iodine from thyroid tissue and in the same year he showed that this iodine was intimately associated with thyroid activity. By acid or peptic hydrolysis of thyroid tissue, a fraction, rich in iodine and in which the whole activity of the thyroid gland was concentrated, was obtained. Thus was established a direct relationship between iodine and the thyroid gland.

In 1899, Oswald prepared a protein "iodothyreoglobuline" (or thyroglobulin), by extracting the whole gland with saline solution and precipitating the pseudoglobulin with saturated ammonium sulphate solution. This precipitation contained 7.7 per cent iodine and had a molecular weight of about 700,000. The substance was recogniced as being the chief physiologically active component of the thyroid alveolar tissue.

In 1919 Kendall (1919) isolated a crystalline substance from thyroid tissue which exerted the full physiological activity of the glandular extract. This substance contained by weight 65 per cent iodine and an amino group. When Harrington and Barger (1927) synthesised thyroxine, the true molecular structure was fully defined. Extraction procedures indicated that thyroxine was the only thyroid hormone in the circulation (Lainlaw, 1949; Rosenberg, 1951). However, subsequent studies of plasma collected after

administration of radioactive iodine (Gross & Pitt-Rivers, 1952) revealed a second biologically active hormone which was identified as triiodothyronine. It is physiologically 4 to 5 times as active as thyroxine and acts more immediately than thyroxine.

There is now a voluminous literature of the iodine-containing compounds of thyroid tissue and those circulating in the plasma.

Anatomy and Physiology

The thyroid is an endocrine gland producing the hormones triiodothyronine (T_3) and thyroxine (T_4). It contains a second endocrine system producing calcitonin, and this in turn has an important role in calcium metabolism. Calcitonin is however outwith the bounds of this present work.

The normal thyroid gland is a bi-lobed symmetrical, firm, smooth organ. The right lobe is often, as a normal variant, larger than the left lobe especially in children. The gland is situated in front of the thyroid cartilage to which it is closely attached by the pretracheal fascia. Because of its anatomical location, good information can be gained by physical examination.

All developmental variations are ultimately associated with hypofunction, due to the sheer quantitative insufficiency of thyroid tissue.

The function of the thyroid gland is to synthesise, store and secrete the two thyroidal hormones viz. triiodothyronine and thyroxine. The metabolic activity of all tissues is influenced by these thyroid hormones, but the precise biochemical mechanisms

involved are uncertain. From the physiological point of view mental development, skeletal growth and sexual maturation all require normal thyroid function. The heart rate and cardiac output are influenced by the thyroid hormones, these potentiating the effects of adrenaline and nor-adrenaline.

Biosynthesis of Thyroid Hormones

The thyroid hormones are synthesised in the gland from iodine and tyrosine (Figure 1). They are then bound to thyroglobulin for storage. Before they are discharged into the blood there is an enzymatic breakdown of thyroglobulin.

Dietary iodine is readily absorbed from the upper gastrointestinal tract and enters the plasma as inorganic iodide. The plasma iodide is either taken up by the thyroid or excreted by the kidney. Although small amounts of iodide are found in the salivary, sweat and mammary glands, stomach and small intestine, only the thyroid gland has the ability to elaborate fully the thyroid hormones.

The remarkable ability of the thyroid to trap iodide is shown by the maintenance of a concentration gradient between the thyroid gland and plasma, usually in a ratio of 20:1 (Pochin, 1950; Schachner et al., 1944). This phenomenon is oxygen-dependent (Freinkel & Ingbar, 1955) and is almost certainly under enzymatic control. Trapping is increased by thyrotrophin (TSH) and decreased by an excess of circulating free iodide, thiocynate and by perchlorate both of which compete with iodide for the transport

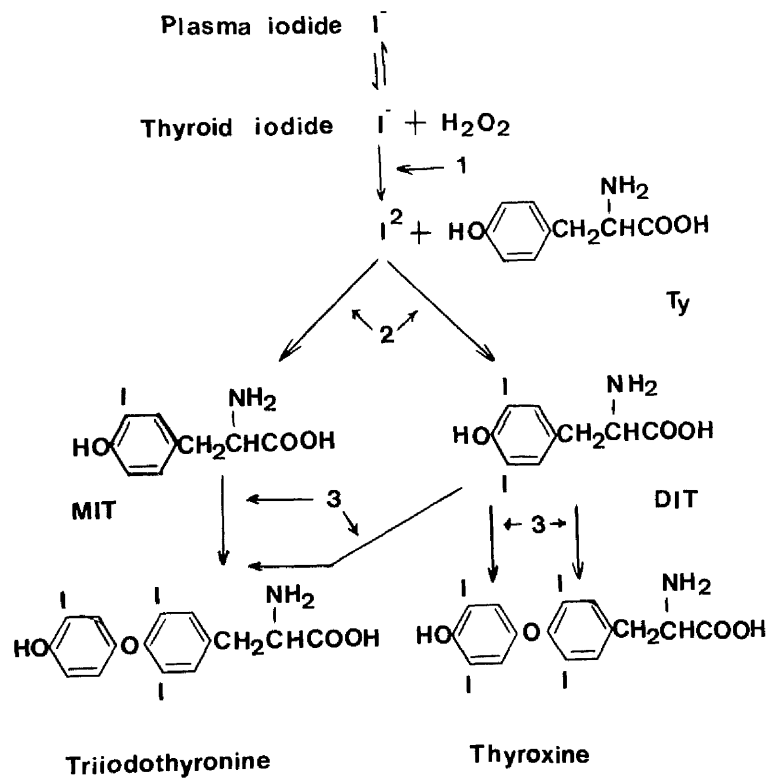


Figure 1 : Schematic representation of steps in the biosynthesis of thyroid hormones

Enzyme legend: 1 = Peroxidase; to oxidise iodide to free elemental iodine.

2 = Iodinase; for iodination of tyrosine to 3-monoiodityrosine and 3,5-diiodotyrosine.

3 = Enzyme coupling 1 mol. MIT and 1 mol. DIT with alanine residue (CH_3-$CH(NH_2)COOH$) (not shown), with the formation of 3,5,3-triiodothyronine.

3' = Enzyme coupling 1 mol. DIT with 1 mol. DIT with alanine residue (not shown), with the formation of 3,5,3',5'-tetraiodothyronine (thyroxine).

Compound legend: Ty = Tyrosine
MIT=3-Monoiodotyrosine
DIT=3,5-Diiodotyrosine

system.

Before the intrathyroidal iodide can be incorporated into the tyrosine molecule it must be oxidised to free elemental iodine. The process of oxidation is catalysed by an enzyme peroxidase, in the presence of hydrogen peroxide. Organification of iodine normally occurs very rapidly in vivo, so that it is possible that another enzyme, tyrosine iodinase is involved (Hutchinson, 1969). In certain situations however this does not take place e.g. congenital biosynthetic defects, autoimmune thyroiditis and following therapy with thiourane drugs. The trapped iodine may be discharged by the administration of perchlorate. Organification leads to the formation of monoiodotyrosine and diiodotyrosine.

Thyroxine is believed to be formed by the coupling of two molecules of diiodotyrosine under the influence of a coupling enzyme, with loss of an alanine side chain from one molecule (Harrington, 1944). Triiodothyronine is formed on the same assumption, by one molecule of monoiodotyrosine and one of diiodotyrosine (Roche & Michel, 1955) or from partial deiodination of thyroxine in the peripheral tissue. The coupling takes place within the thyroglobulin, but the precise nature of the mechanism is not known. In the congenital coupling defect, monoiodotyrosine and diiodotyrosine may appear in the blood (Floyd et al., 1960).

The thyroid hormones are stored within the thyroglobulin until required by their target organs. Then thyroglobulin is digested by thyroid proteases and peptidases. Proteolysis of thyroglobulin results in the liberation of the iodotyrosines (monoiodotyrosine and diiodotyrosine) as well as the iodothyronines (triiodothyronine and thyroxine). The iodotyrosines unlike the

iodothyronines do not normally enter the blood stream because they are deiodinated within the gland by the enzyme dehalogenase and that iodine is re-utilised for the formation of thyroid hormones. Deficiency of dehalogenase leads to the escape of monoiodotyrosine and diiodotyrosine into the circulation and subsequent loss in the urine thus leading to iodine deficiency.

Control of Thyroid Hormone Secretion

The concentration of thyroid hormones in blood is controlled by a dynamic control system involving the hypothalamus, the pituitary and the thyroid gland.

Thyrotrophin, (TSH, thyroid-stimulating hormone) which is secreted by specific pituitary cells is the major regulator of thyroid hormone formation and release. Thyrotrophin is secreted under the influence of a tripeptide, thyrotrophin releasing hormone (TRH) formed in the hypothalamus and transported to the anterior pituitary via the portal vessels. The effect of thyrotrophin releasing hormone on thyrotrophin secretion is inhibited by the thyroid hormones. Enhanced peripheral utilisation of thyroid hormones leads to reduction of the feedback inhibition of thyrotrophin releasing hormone, this action resulting in further thyrotrophin secretion and thyroxine formation to restore the blood level to equilibrium.

Thyroid Hormones in the Circulation

The bulk of the circulating thyroid hormones is thyroxine (60-150 nmol. per litre) with only 1-2 per cent as triiodothyronine (1.0-2.6 nmol. per litre). The hormones are bound to specific binding proteins in the plasma with only a small fraction in the free or unbound form. The major binding proteins are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and albumin (TBA) which carry 60, 30 and 10 per cent respectively of the approximate total circulating thyroxine. While triiodothyronine is also carried by proteins, there are major differences in the binding between the two hormones. Triiodothyronine is not bound by thyroxine-binding prealbumin and only weakly by thyroxine-binding globulin and albumin (Larson & Albright, 1965; Robbins & Rall, 1955). However, only the free, unbound hormone is able to exert a metabolic effect (Robbins & Rall, 1960; Sterling & Hegedus, 1962). Free thyroxine is about 0.05 (25-50 pmol per litre) and free triiodothyronine 0.5 per cent (6-11 pmol per litre) of the respective total circulating thyroid hormones. The binding proteins act as buffers in the extrathyroidal metabolism, circulating the store of thyroid hormones and preventing their early loss in the urine.

Catabolism of Thyroid Hormones to their Excretory Products

The peripheral degradation of thyroid hormones proceeds through several metabolic pathways, chiefly deiodination, conjugation with sulphate or glucuronic acid and side-chain metabolism (Figure 2).

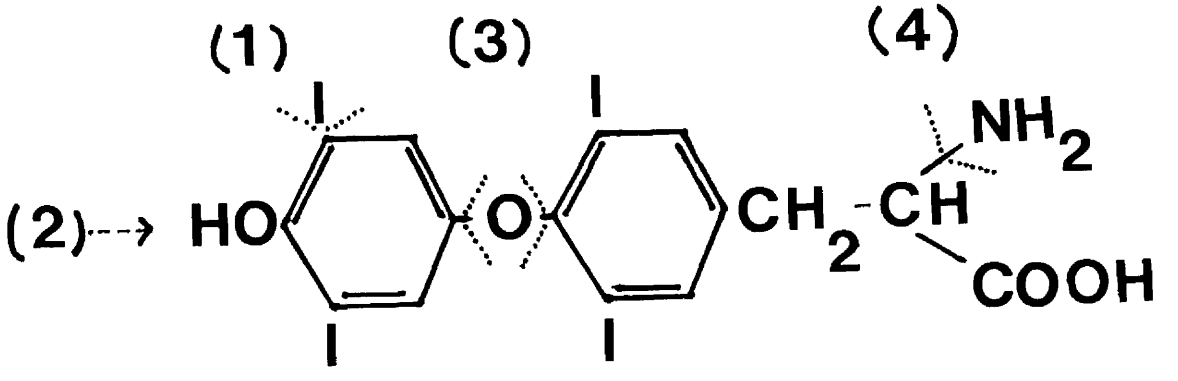


Figure 2 : Biochemical transformations of the thyroid hormone molecules in the body tissues ; (1) deiodination, (2) conjugation of the phenol, (3) rupture of the diphenyl ether linkage and (4) oxidative deamination of the alanine side chain.

The daily degradation of triiodothyronine and thyroxine has been determined as approximately 60ug. for triiodothyronine and 80ug. for thyroxine (Hoffenberg, 1973).

Deiodination is the most important metabolic pathway of thyroid hormones. After intravenous administration of radioiodine-labelled thyroxine to normal man, less than 10 per cent of the radioiodine in the urine is present as organic iodine (Pittman et al., 1972), the other 90 per cent being present as inorganic iodide.

Deiodination by iodothyronine deiodinase effectively deiodinates triiodothyronine and thyroxine, but not iodotyrosines (Plaskett, 1962). By deiodination, iodine released in the circulation is in part taken up by the thyroid and in part excreted as iodide in the urine. This pathway has been demonstrated in numerous tissues, such as liver, kidney, muscle, heart and brain (Albright et al., 1959; Etting & Barker, 1959). Only a proportion of the total amount of triiodothyronine produced daily is actually secreted directly from the thyroid gland, the remainder is derived from peripheral monodeiodination of thyroxine. In fact approximately one-third of the total circulating thyroxine is converted to triiodothyronine (Pittman et al., 1971). Complete deiodination of thyroid hormones may also occur, leaving only the thyronine nucleus (Pittman et al., 1972). In addition to thyronine, deiodinated metabolites of thyroxine appear in urine. These are monoiodotyrosine and diiodotyrosine (Roche et al., 1952 (a and b)).

Similarly triiodothyronine is deiodinated to 3.3'-diiodothyronine and is its major metabolite appearing in the urine (Folk et al., 1960).

Administered radioactive triiodothyronine appears to some extent as monoiodotyrosine and diiodotyrosine in the urine (Roche et al., 1952 (a and b). These steps are summarized in Table I and Figure 3. The compartments in which the hormones are distributed are shown.

Conjugation (Table I) of the phenolic hydroxyl group of thyroxine and triiodothyronine with glucuronic acid or sulphate occurs in the liver and kidneys (Folk et al., 1960; Etting & Barker, 1959). The resulting hepatic glucuronosides and etheral sulphates are either excreted in the bile and undergo intestinal hydrolysis (β-glucuronidase) before reabsorption, or are returned to the circulation as conjugates for excretion in the urine. Most of the freed hormones or their derivatives in the gut are reabsorbed into the circulation (enterohepatic cycle) and only a small portion is excreted in the faeces. Burke et al. (1972) found 0.65 and 3.69ug. per day respectively of triiodothyronine and thyroxine conjugates in the urine (Table II).

Oxidative deamination (Table I) and decarboxylation of the alanine side-chain to their acetic acid derivatives is the predominant metabolic pathway for thyroid hormones in the kidney (Pittman et al., 1972). Tetra- and tri-iodothyroacetic acid have also been found in liver and brain, and the pyruvic and lactic acid derivatives of triiodothyronine and thyroxine have been demonstrated in both urine and bile. The average daily excretion of thyroacetic acid in urine, from thyroxine origin, is approximately 7.5ug. per day (Pittman et al., 1972). Administered thyroxine may also appear in urine as tetraiodothyropropionic acid (Roche et al., 1954).

Finally, excretion of the intact hormones in the urine is

TABLE I : Catabolism of Thyroid Hormones to their Excretory Products (in vivo)

Catabolic event	Metabolites Identified	Principal Organs Involved	Principal Compartment for Excretion
(1) Deiodination	Iodide, Monoiodotyrosine, Diiodotyrosine, Thyronine, Diiodothyronine, Triiodothyronine.	Liver and kidney; other tissues to lesser extent.	Urine, in small amounts in most tissues.
(2) Conjugation of the phenol nucleus			
(a) with glucuronic acid	Glucuronides of iodothyronines (Diiodothyronine, Triiodothyronine, Thyroxine)	Liver, kidney	Bile, small amounts in urine
(b) with sulphate	Sulphate ester (Triiodothyronine)	Liver	
(3) Rupture of diphenyl ether bond	Diiodotyrosine	Not identified	Urine
(4) Deamination	Acetic and pyruvic acid analogues of Thyroxine and Triiodothyronine	Liver, kidney.	Bile, kidney, liver, urine

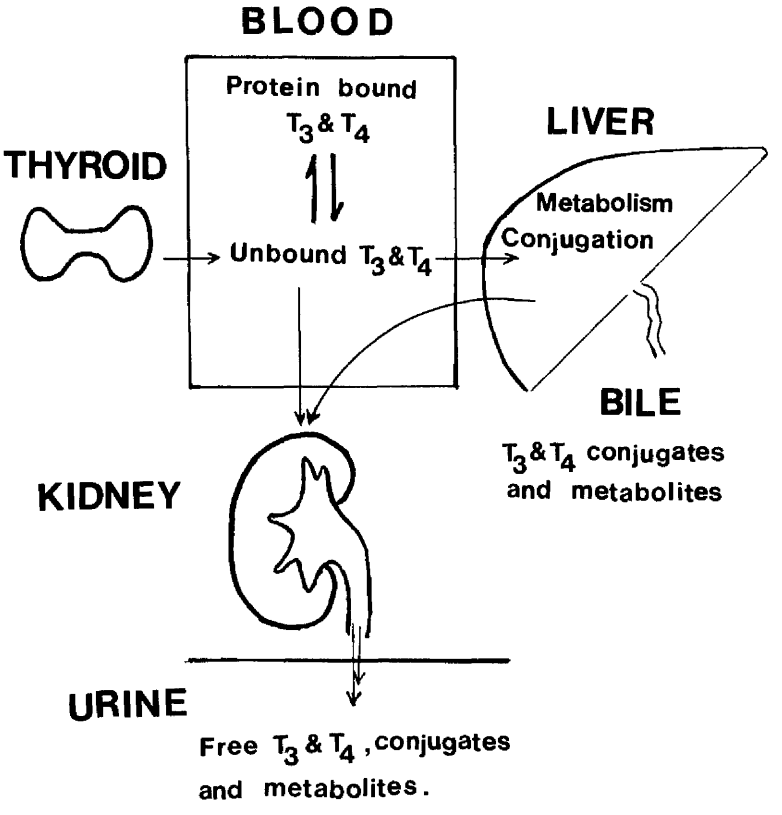


Figure 3 : Progress through body compartments of the thyroid hormones.

TABLE II : Unconjugated, conjugated (pH 1 hydrolysable and total triiodothyronine and thyroxine in urine (mean ug. per 24-hour and percentage). Review of the literature.

Authors	Triiodothyronine			Thyroxine		
	Unconjugated	Conjugated	Total	Unconjugated	Conjugated	Total
Burke et al., 1972	0.71 (52%)	0.65 (48%)	1.36	2.36 (39%)	3.69 (61%)	6.05
Gaitan et al., 1975	1.57 (77%)	0.47 (23%)	2.04			
Pastrana et al., 1974				2.10 (40%)	3.10 (60%)	5.20

presumably because of filtration of free plasma thyroxine and triiodothyronine (Chan et al., 1972) which is in equilibrium in the circulation with the other moities already mentioned.

It is these free hormones which are of interest for this present work because generally it is accepted that the free hormones are the metabolically active fraction. If their quantity in the urine could be reliably measured, some indication of thyroid function may thereby be adduced. However the value of such a measure would be vitiated if the free thyroid hormones were significantly reabsorbed in the renal tubule. This problem is now considered.

Renal Handling of Thyroid Hormones

Uptake

Renal uptake of triiodothyronine was studied by Cavalieri et al., (1975). They monitored the accumulated radioactivity over the kidney area during a sustained intravenous infusion of ^{131}I-labelled triiodothyronine given over 50 to 90 minutes. There was an accumulation of 25ng. per minute. They concluded, after comparing their renal triiodothyronine uptake values with those of unbound urinary triiodothyronine in health (Burke et al., 1972) (approximately 0.5ng. per minute) that only about 2 per cent of the renal uptake material appeared in the urine, the remainder being degraded within the kidney or returned to the plasma as unchanged hormone.

Clearance

Thyroxine

Studies by Burke et al., (1972) on unbound serum thyroxine indicated a mean clearance of free thyroxine in normal subjects of 26ml. per minute. In another study Burke & Shakespear (1975) (Table III) reported a clearance of 38ml. per minute for free thyroxine. These values suggest that unbound thyroxine is ultrafilterable, but reabsorbable by the tubule. These authors have calculated precisely the tubular reabsorption of thyroxine and found it to be 65 per cent. In thyrotoxic patients, the mean thyroxine excretion is increased 7.5 fold, while serum unbound thyroxine is increased by only 3-fold. This suggests a fall in tubular reabsorption. Indeed the clearance value of thyroxine increases by 26 per cent to 48ml. per minute. The implication therefore is that there might be a maximum tubular reabsorption rate for thyroxine. In hypothyroidism, there is also an increase in the mean apparent thyroxine clearance, but the range is very wide (55 to 270ml. per minute). Assuming that the thyroxine clearance is approximately one-third of the creatinine clearance (creatinine clearance 120ml. per minute, thyroxine clearance 38ml. per minute), some 70 per cent of the filtered free thyroxine is therefore reabsorbed. The remaining 30 per cent appears in the urine as free thyroxine.

TABLE III : Urinary clearances of triiodothyronine and thyroxine of adults quoted by Burke & Shakespear (1975).

	Clearance (ml. per minute)	
Group examined	Triiodothyronine mean ± S.D.(n°)	Thyroxine mean ± S.D.(n°)
Euthyroid	164 ± 80 (12)	38 ± 21 (12)
Hyperthyroid	221 ± 80 (10)	48 ± 22 (11)
Hypothyroid	174 ± 72 (17)	138 ± 71 (7)

The degree of tubular reabsorption of thyroxine calculated at about 65 per cent, contrasts with the more complete reabsorption of unconjugated cortisol (98 per cent) (Beisel et al., 1964), which has a clearance rate of about 10ml. per minute (Burke & Beardwell, 1973). One probable factor responsible for this difference could be the much smaller amount of thyroxine compared to cortisol in urine relative to the normal amounts of protein present in the urine. Protein-binding of the hormones in the glomerular filtrate might reduce tubular reabsorption. The very high affinity of thyroxine for protein could in part be responsible for the poor reabsorption. As water reabsorption proceeds in the tubule the effect of protein might begin to retard thyroxine reabsorption.

This effect may normally be small, but might be large in cases of proteinuria. More significant normally may be the binding of thyroxine to other molecular species in the tubular fluid, for example, by the low molecular weight binder whose presence in urine is suggested by Burke & Shakespear (1976).

This problem of the extent to which the tubular reabsorption of thyroid hormones is affected by urinary proteins and other low molecular weight substances occurring in the urine has been resolved by the work of Burke & Shakespear (1976). They have shown that of the total normal urinary thyroid hormones, 40 per cent triiodothyronine and 20 per cent thyroxine are free, up to 9 per cent triiodothyronine and 12 per cent thyroxine are protein-bound, and about 50 per cent of urinary triiodothyronine and 68 per cent thyroxine are associated with a low molecular weight substance. This is shown in Table form (Table IV).

TABLE IV : Percentage binding of unconjugated triiodothyronine and thyroxine in urine (Burke & Shakespear, 1976).

	Mean T_3(%)	Mean T_4(%)
Bound (to protein plus "LMW binder")	60.6	79.9
Bound to protein	8.9	11.9
Bound to "LMW binder"	51.7	68.0
Unbound (Free)	39.4	20.1

From the above, it is clear that factors controlling the tubular reabsorption of thyroxine are complex. Urinary thyroxine excretion is markedly increased by high protein excretion rates due to the carriage of more serum-protein-bound thyroxine through the glomeruli into the urine. This is seen in the nephrotic syndrome. For the same reason, the ostensibly high thyroxine clearance rates noted in some hypothyroid subjects is due to increased protein excretion and possibly also thereby to reduction in tubular reabsorptive activity. This has been referred to as "renal hypothyroidism". Another possible explanation for the apparently increased thyroxine clearance rates seen in hypothyroidism is the overestimation of hormone because of cross reaction with the metabolites of thyroxine (thyroacetic and thyropyruvic acid) which is more significant at low urinary concentrations of thyroxine.

Triiodothyronine

While thyroxine clearance in euthyroid subjects was thus about one-third of creatinine clearance, apparent triiodothyronine clearance was 1.5 times higher than the creatinine clearance (164 ± 80 ml. per minute) with a wide range (Burke & Shakespear, 1976), and with no significant difference from hypothyroid subjects (174 ± 72 ml. per mimute). However, triiodothyronine clearance was a little higher in thyrotoxic subjects (221 ± 80ml. per minute).

In terms of the excreted mass of triiodothyronine, the reported values are sufficiently high when compared with the plasma levels to indicate that it is almost certain that not only is the clearance

rate of triiodothyronine high but there is possibly also an active secretion of triiodothyronine at tubular level. Burke & Shakespear (1976) have indeed shown that the triiodothyronine clearance rate is 164 ± 80ml. per minute which is some 4-5 times higher than that of thyroxine. They found in cases of hypothyroidism clearance rates of 174 ± 72ml. per minute and in thyrotoxicosis values of 221 ± 80ml. per minute. Even these high clearance values cannot fully account for the high urinary content of triiodothyronine and thus an active tubular excretion of triiodothyronine seems likely.

By comparing the mean filtered load of triiodothyronine presented to the tubules in normal subjects (450 pg. per minute) with the mean urinary excretion rate (570 pg. per minute) Burke & Shakespear (1976) have estimated that 21 per cent of the urinary triiodothyronine does not come from glomerular filtration of serum unbound triiodothyronine alone but from an active tubular excretion of triiodothyronine. This triiodothyronine might well represent the end product of thyroxine, which has been reabsorbed or delivered actively by the circulation to the tubule but metabolised in the tubular wall to triiodothyronine (Gaitan et al., 1975).

Another possible reason for the mean apparent triiodothyronine clearance being about 1.5 times the creatinine clearance is that urinary triiodothyronine is overestimated by the radioimmunoassay methods used. Several thyroid hormone metabolites in human urine, not all of which have been fully identified (Pittman et al., 1972) cross react with the antibody (Hufner & Hesch, 1973) of the method.

The renal clearances of triiodothyronine and thyroxine are poorly correlated with creatinine clearance (Burke & Shakespear, 1975).

However in general the lower the creatinine clearance the lower the thyroid hormone clearance, with this exception that there is an elevated clearance of thyroxine in hypothyroidism when the creatinine clearance is low. Urinary triiodothyronine is underestimated in subjects with a creatinine clearance of less than 50ml. per minute. In general, assay of urinary triiodothyronine is invalidated in patients with acute or chronic renal failure who have reduced glomerular filtration (Chan, 1974) and whose creatinine clearance falls below 30 to 50ml. per minute (Burke, 1974).

It is not yet clear what is the contribution of renal metabolism of these hormones to the values estimated. The kidney plays an important role in the peripheral metabolism of iodine and thyroid hormones and the thyroid hormones play an important role in kidney growth and function (Katz et al., 1975). Both hypo- and hyperthyroid states are accompanied by numerous renal functional abnormalities. In uncomplicated thyrotoxicosis the glomerular filtration rate, the renal plasma flow, the tubular reabsorptive and the tubular secretory ability are usually increased. In thyroid deficiency, the renal functional alterations are generally the opposite. Thus the final figures represent the end product of equations which include secretion, liver metabolism, protein-binding, target organ utilization and renal handling.

Relationship of Urine Thyroxine and Triiodothyronine to Serum Unbound Levels

The relationship between urinary thyroid hormones and their serum unbound fractions have been found to be linear over a wide

range of hormone concentration (Burke & SHakespear, 1975). This relationship is maintained irrespective of the changes in the circulating levels of thyroid-binding-proteins or their binding capacity. For example, in pregnancy and during oestrogen therapy (Chan, 1974; Burke & Shakespear, 1975; Gaitan et al., 1975; Pastrana et al., 1975) where the total thyroid hormone levels are elevated due to a rise in thyroxine-binding globulin the thyroid hormone excretion rate is normal as are also the unbound serum levels. Conversely, in patients with a low concentration of thyroxine-binding globulin (idiopathic low TBG, hypoproteinaemia, massive corticosteroid therapy) and altered thyroxine-binding capacity (salicylate ingestion or phenytoin), thyroid hormone excretion rates are normal despite low total serum levels (Chan, 1974; Burke & Shakespear, 1975; Pastrana et al., 1975), because here also the serum unbound levels are normal.

From these facts it can be appreciated that there is some relevance in studying the urinary excretion rates of the thyroid hormones since these rates reflect the unbound serum concentrations of the hormones. However, the quoted values by different groups (Table V) differ significantly.

TABLE V : Values of urinary thyroid hormones (ug/24h) in euthyroid adults and in disease states (mean ± S.D.(n)) as recorded in the literature.

TRIIODOTHYRONINE

	Chan et al., 1972	Burke & Shakespear, 1975	Rastogi et al., 1974*
Euthyroid	2.9 ± 0.5 (44)	1.11 ± 0.4 (44)	7 (10)
Pregnant	3.3 ± 0.8 (24)	1.05 ± 0.45 (22)	10 (10)
Hyperthyroid	9.3 ± 3.8 (17)	6.74 ± 5.08 (46)	
Hypothyroid	0.9 ± 0.5 (12)	0.44 ± 0.75 (16)	

THYROXINE

	Chan et al., 1972	Burke & Shakespear, 1975	Rastogi et al., 1974*
Euthyroid	8.0 ± 2.1 (44)	1.97 ± 0.82 (38)	13 (10)
Pregnant or oral contraceptive	8.1 ± 2.0 (24)	1.61 ± 0.79	12 (10)
Hyperthyroid	19.3 ± 8.8 (17)	8.08 ± 5.42 (42)	
Hypothyroid	2.8 ± 0.9 (12)	0.78 ± 0.9 (16)	

* Calculated from graphic data supplied by Rastogi et al., (1974).

Current Techniques for the Measurement of Thyroid Hormones in Urine

Competitive Protein Binding Method (CPB)

In the earliest method reported (Chan & Landon, 1972) acid urine was extracted with ethyl acetate and the evaporated extract was subsequently assayed by a competitive protein binding method. A mean value for the urinary excretion of unbound thyroxine in euthyroid subjects was found to be 8ug. per 24 hours with higher and lower values for hyperthyroid and hypothyroid patients respectively. Much lower values were reported by Burke et al., (1972), who extracted urine on Sephadex columns. This disparity was partly explained by the inclusion of compounds which compete in the protein binding system resulting from the ethyl acetate extraction (Black et al., 1973) and the hydrolysis of the hormone conjugates by the acidification of the urine (Chan, 1974). In general, most workers (Shakespear & Burke, 1976; Black et al., 1975) agree that competitive protein binding assay methods give higher thyroxine values compared to those measured by radioimmunoassay techniques. It is concluded that some of the many thyroxine metabolites are measured by the competitive binding assay system because quite a number of thyroxine peptide derivatives can displace thyroxine from thyroxine binding globulin (Tabachnick et al., 1971). Black et al. (1975) have run a comparative study of competitive protein binding and radioimmunoassay as a means of measuring urine thyroxine (Table VI) and concluded that the competitive protein binding method overestimated the true value of

TABLE VI : Comparison of the competitive protein binding and radioimmunoassay methods for urinary thyroxine performed on the same samples (Black et al., 1975)

Adult Group examined	Radioimmunoassay (ng. per 24 hours)			Competitive protein binding (ng. per 24-hours)
	Triiodothyronine	Thyroxine	Triiodothyronine + Thyroxine	Thyroxine
Euthyroid	1.0 ± 0.35	1.23 ± 0.49	2.23 ± 0.71	2.0 ± 0.63
Hyperthyroid	4.5 ± 2.63	5.57 ± 5.39	10.1 ± 7.7	6.1 ± 3.5

thyroxine by 60 per cent. Further this overestimation could not have been due to measurement of triiodothyronine as the authors concluded that only 10 per cent of the urinary triiodothyronine is measured as thyroxine by the competitive protein binding technique. In hyperthyroid subjects urinary thyroxine assayed by a competitive protein binding technique was considerably less than the sum of urinary triiodothyronine and thyroxine measured by radioimmunoassay. Thus competitive protein binding methods for urinary thyroxine overestimates the true value.

Radioimmunoassay Methodology (RIA)

With this method there is a disparity between the values reported by workers and they regard cross reaction between the hormone metabolites in urine as the main defect of the method largely because the respectively raised antibodies bind variously different metabolites quantitatively. This later observation is demonstrated in the excessively high normal values for urinary triiodothyronine and thyroxine reported by Rastogi et al., (1974) who employed radioimmunoassay on unextracted urine even although these workers excluded significant cross reaction with a number of hormone conjugates and metabolites in their assay system. Shakespear & Burke (1976) examined the effect of storage of urine at alkaline pH (by addition of sodium bicarbonate as preservative) under different temperature conditions. Although their mean results (Table VII) suggest that triiodothyronine and thyroxine are more stable in urine stored at $-20^{\circ}C$ compared to those stored at $4^{\circ}C$

TABLE VII : Effect of storage of urine at various temperatures on thyroxine concentrations measured by radioimmunoassay (Shakespear & Burke, 1976).

Days of Storage / Temperature	Hormone found as percentage of that found in fresh urine. Mean of 8 urines (and range)		
	23°C	4°C	-20°C
0	100%	100%	100%
4 days	111.9% (101.4-133.3)	102.4% (86.5-115.2)	104.5% (97.9-111.4)
8 days	160.7% (117.4-221.1)	119.5% (104.3-150.1)	124.8% (111.1-139.8)
14 days	104.9% (41.7-165.9)	119.8% (106.5-131.9)	113.8% (102.2-122.9)
36 days	—	129.9% (115.7-150.6)	125.5% (115.7-141.5)
123 days	—	—	106.9% (95.6-113.3)

and at room temperature (23°C), values found for individual urines were unpredictable even for those stored at -20°C, some rising and some falling. Additionally with increase in storage some urinary thyroxine levels rose linearly while triiodothyronine levels decreased linearly. Wilkinson & Bowden (1960) have shown that iodothyronines decompose at room temperature in alkaline pH after two days.

These data question the specificity of the current radioimmuno-assay methods and confirm the doubt of other workers (Gaitan et al., 1975) that by RIA the ultrafiltrate, the conjugates and the metabolites of the thyroid hormones should be assayed as well.

Excess proteinuria is another complicating factor (Burke et al., 1973). Burke & Shakespear (1975) found that urinary thyroxine was overestimated in an RIA system when the protein excretion rate was greater than approximately 150mg. per day. In the same report they showed that the level of triiodothyronine was insignificantly affected by physiological amounts of proteinuria while a little increase in protein (250mg. per day) excretion led to under-estimation of triiodothyronine. Thus when the triiodothyronine level in urine is low (as in athyreosis) a small amount of protein in the urine will exert a relatively greater effect on the radioimmunoassay system than when the triiodothyronine level is high (Gaitan et al., 1975). Also, the urinary thyroid hormone assay by the radioimmunoassay method is of limited value in patients with more than physiological proteinuria. In the nephrotic syndrome there is a considerable loss of thyroxine-binding proteins and protein-bound hormonal iodine (Rasmussen, 1956), and it can be appreciated that lesser degrees of

proteinuria are not without their effects on a radioimmunoassay system. For thyroxine-binding globulin has a molecular weight 59,000 and albumin a molecular weight of 69,000 and therefore both are readily lost to urine when the pathology permits. In a recent report (Finucane et al., 1977) euthyroid patients with renal disease were shown to have a renal loss of thyroxine estimated by radioimmunoassay greater than the average normal daily loss by ten-fold, whereas in the same patients the loss of triiodothyronine was slightly less than the normal range and indeed the levels were in the range found in hypothyroid patients.

Radioimmunoassay methods therefore although technically simple and expeditious are disadvantaged by factors beyond the investigator's control.

Proposed Methodology

Gas/Liquid Chromatography of Urinary Thyroid Hormones

The iodotyrosines and iodothyronines, by virtue of their attached halogen groups are ideally constructed molecules for activation against the electron capture detection of the gas liquid chromatography system. The use of gas liquid chromatography as a method for determinating the level of thyroid hormones in biological fluids, would have obvious advantages. There is a high resolving power, the clean separation of triiodothyronine from thyroxine and the ultrasensitive electron capture detection technique for the final quantitation of the very minute physiological amounts

of thyroid hormones as might be expected in the urine.

If a gas liquid chromatography method were practical for estimation of free urinarytriiodothyronine and thyroxine then clearly linkage to a Mass-Spectrometer would help to detect and identify compounds and their quantitation at femtomole level. This present work aims at establishing a gas liquid chromatography method and studies in this direction may be of help to better understanding of thyroid metabolism.

Already both flame ionisation detection (FID) and electron capture detection (ECD)(^{63}Ni) have been used successfully in the analysis of derivatives of thyroid related iodoaminoacids (Jaakonmaki & Stouffer, 1967). While various authors have confirmed the practical nature of this form of quantitation, I have been unable to find published work on the application of the gas liquid chromatography methodology for the quantitation of thyroid hormones extracted from urine. It is here that I pioneer a suitable procedure.

CHAPTER 2

MATERIALS AND METHODS

Introduction

In this thesis I intend to present data on the urinary concentration of the active thyroid hormones estimated by a method utilising gas liquid chromatography for the final quantitation. I have chosen such a system because the use of radioactivity is not absolutely essential. Although here I have used trace amounts of radioactively labelled thyroxine in recovery experiments and to test the reliability and reproducibility of the method, if the method satisfies accepted criteria, then the omission of repeated recovery experiments in certain circumstances may be permissible. In Cairo, where I shall subsequently be practising, facilities for the purchase and quantitation of radioactivity are not freely available.

In the following pages I describe my methodology.

Materials

Chloroform, ethyl acetate, isopropanol, methanol, methyl acetate, triethylamine, trimethylpentane (isooctane), ammonia solution, ammonium acetate, formic acid, hydrochloric acid, and sodium hydrogen bicarbonate were obtained from British Drug Houses (BDH) Chemicals Ltd., Poole, England, and were whenever available of "Analar" specification. Hydrochloric acid was obtained in two forms viz. concentrated liquid and hydrogen chloride gas (99.6 per cent)

(0.5 lb) contained in monel metal cylinders equipped with an adaptor and control valve.

Dry methanol (completely water-free) and dimethyldichlorosilane were purchased from Applied Science Laboratories, State College, U.S.A. and were used without further treatment.

Toluene (scintillation grade) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire. Both PPO (2,5-diphenyl-oxazole) and POPOP (1,4-di(2-(5-phentloxozolyl)-benzene)) were obtained from Hopkin & Williams Ltd., Redding Industrial Estate, Falkirk. From the same firm I obtained rice starch.

Ninhydrin spray (0.5 per cent in n-butanol) was purchased as an aerosol from May and Baker Ltd., Dagenham, Essex. Trimethylacetic anhydride (pivalic anhydride) (99 per cent pure by g.l.c.) was bought from Aldrich Chemical Co. Ltd., 264 Water Road, Wembley, Middlesex.

Merk silica gel F_{254} (type 60) was purchased from Anderman & Co. Ltd., Central Avenue, East Molesey, Surrey.

OV-17 (phenylmethylsilicone) and high performance (HP) Gas Chrom Q mesh 100-200 were obtained from Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire.

The iodinated aminoacids; 3-monoiodotyrosine, 3,5-diiodotyrosine, 3,5-diiodothyronine, 3,5,3'-triiodothyronine and thyroxine were obtained as the free acids from Sigma (London) Co. Ltd., Norbiton Station Yard, Kingston-Upon-Thames, Surrey. From the same firm I obtained Dowex 50W-X2 (H^+) mesh 100-200.

Thyroxine-2-^{14}C (side-chain label) S.A. 30.4 mC/mM was supplied by New England Nuclear, Albany Street, Boston, U.S.A.

Silanisation of Glassware

The thyroid hormones tend to be adsorbed to glassware (Lee & Pileggi, 1971). When low urinary levels are the order, this adsorption could create significant problems in terms of accuracy and reproducibility of the method. Clean glassware surfaces were therefore ensured by rinsing all glassware for 15 minutes at room temperature with dimethyldichlorosilane (5 per cent) in toluene, followed by a rinse with methanol. After drying in air the glassware was dried in an oven for 4 hours (140°C). This silanisation procedure was used throughout the entire work on all glassware which would be intimately in contact with the thyroid hormones. The dimethyldichlorosilane effectively reacts with active sites on glass surfaces, i.e. converting the polar silanal groups into more inert silyl ether groups. Additionally the methanol inactivates any remaining chlorine atoms. These procedures together with the prolonged heating give the glass surfaces the desirable feature of 'inertness'.

Preparation of Solutions and Substances

Working solutions of ammonium hydroxide (0.5; 2.0; 5.0 and 7.5 molar solutions) were prepared from ammonia solution (AR) (s.g. 0.88), by adding respectively 28; 112; 320 and 420ml. to 1 litre of deionised water.

Hydrochloric acid (1.0 and 5.0 molar solutions) were prepared from concentrated hydrochloric acid (AR) (s.g. 1.18), by adding

respectively 86 and 430 ml. to 1 litre of deionised water.

Ammonium acetate (0.15 molar solution; pH 8.5) was prepared by dissolving ammonium acetate (AR) (11.6g) in 1 litre of deionised water. The pH was adjusted as necessary by the addition of sodium hydroxide (5.0 molar solution).

Sodium hydroxide (5.0 molar solution) was prepared by dissolving sodium hydroxide (AR) (40 g) in freshly boiled and cooled deionised water (200ml.). The solution was stored in a glass bottle fitted with a rubber stopper.

Deionised water was prepared by a water purification apparatus (Elgastat; Model B. 102, Elga Products Ltd., England). Deionised water was de-gassed by boiling followed by cooling, prior to use in the preparation of all aqueous solutions for and in column chromatographic procedures.

Saturated hydrochloric acid/methanol solution (25 per cent w/v) was prepared according to Tajuddin & Elfbaum (1973) although modified as under. Dry methanol (10 ml.) was pippetted into a dry centrifuge tube (25 ml.) and weighed. The tube was then placed in an ice-bath in the fume cupboard and a stream of hydrogen chloride gas bubbled through the methanol for approximately 15 minutes. Thereafter the tube was removed from the ice, rapidly dried and weighed. This procedure was repeated until an approximate 25 per cent increase in the test-tube weight was observed (usually after 15 minutes). This solution was always prepared immediately before use.

Pivalic anhydride (trimethyl acetic anhydride) was used as supplied. Upon receipt, it was divided into 1 ml. aliquots and stored in small brown vials sealed under nitrogen. The vials were

stored in the dark at -4°C. The reagent was used within one month (Nihei et al., 1971) of purchase.

Solutions of the iodoaminoacids* were prepared by the method of Pitt-Rivers & Schwartz (1967). Thyroxine (5mg.), triiodothyronine (5mg.), diiodothyronine (3mg.), diiodotyrosine (3mg.), and monoiodotyrosine (3mg.) were dissolved respectively in methanol/0.5 molar ammonia (2:1, v/v) (1ml.) and stored at 4°C.

Thin Layer Chromatography Plates

These were prepared in the laboratory by adding to deionised water (97ml.) rice starch (0.75 g) and boiling the mixture for 10 minutes. When the starch mixture became translucent, silica gel F_{254} (type 60)** (30 g) was added and homogenised in a blender (top speed : 1 minute). Spreading of plates (20 x 20 cm) was effected by a motorised thin layer coater (0.25mm layers). The plates were then dried in air for 8 hours at room temperature, followed by washing (2 x) in clean methanol. The coated plates were stored dry in glass tanks sealed with wax.

* These compounds are quite insoluble unless the pH is high enough for ionisation of the phenol ring structure.

** Medium-porosity silica gel; mean pore diameter 60 Angstrom units. The added inorganic luminiphore can not be eluted and fluoresces strongly when viewed under short-wave ultraviolet light (maximum 254nm).

Radioactively Labelled Thyroxine

Thyroxine-2-^{14}C (S.A. 30.4 mC/mM) was stored at -20°C as a crystalline solid in a screw-cap bottle. By this procedure there is less than 1 per cent degradation annually (manufacturer's data). Prior to use it was purified by thin layer chromatography as follows:

A small amount of crystalline material (approximately 5.55×10^6 d.p.m.) was dissolved in methanol: 0.5 molar ammonium hydroxide (2:1 v/v) solution (60ul). Samples were quantitatively applied to the thin layer (as prepared) using disposable glass microsampling pipettes, and aided by a thin layer chromatography tray and spotting guide (Baird & Tatlock, London). Rapid drying was effected by a stream of cold air from an air blower (Rapmac Ltd., Watford, Herts.). Unlabelled thyroxine as a reference compound was spotted on side lanes. The plates were developed in several solvent systems until, on radiochromatographic scanning, a single peak of Gaussian form was obtained. The thin layer chromatography tanks were lined with Whatman 3 MM chromatography paper (Reeve Angel & Co., New Bridge Street, London), and sealed with a ground glass lid. The plates were allowed to dry after development.

A single peak of Gaussian form was taken as indication of purity. The solvent systems used were butanol: acetone: 10 molar ammonium hydroxide (50:25:18, v/v/v), methyl acetate:isopropanol: 5 molar ammonium hydroxide (45:35:20, v/v/v) and chloroform: methanol:formic acid (70:15:15, v/v/v). As much as 5 per cent of the radioactivity in the commercially supplied radioactively labelled thyroxine was removed as impurity.

Areas of radioactive thyroxine were located on the thin layer chromatograms using a Panax thin layer radiochromatogram scanner Model RTLS LA (Panax Equipment Ltd., Redhill, Surrey), in conjunction with a Smith's flatbed recorder (Smith's Industries Ltd., Industrial Instrument Division, Kelvin House, Wembley Park, Middlesex). The detector carrier gas was a mixture of argon (2 per cent) and propane (98 per cent) (British Oxygen Company Ltd., Special Gases Department, Deer Park Road, London). The gas flow rate was regulated to 50ml. per minute. The Geiger-Muller detector was adjusted to a height of approximately 1 mm. above the thin layer plate and all plates were scanned using the 15 x 1 mm. detector aperture. The detector voltage was set at 1,040 V; the detector dead time at 200 msec. and the discriminator bias at 10 mV. A time constant of 100 seconds and a scanning speed of 30 mm. per hour was employed. The range of counting varied with the amount of radioactivity to be scanned.

According to peaks of radioactivity obtained by the tracing the Rf value of thyroxine in the solvent systems used is shown in Table VIII.

TABLE VIII : Rf Value of thyroxine-2-^{14}C in three solvent systems

Solvent system	Rf value
Butanol:acetone: 10 molar ammonium hydroxide (50:25:18, v/v/v)	0.70
Methyl acetate:isopropanolol:5 molar ammonium-hydroxide. (45:35:20, v/v/v)	0.59
Chloroform:methanol:formic acid (70:15:15,v/v/v)	0.50

The silica over the area corresponding to the peak traced and to the loci of the cold standard was loosened from the thin layer plate using a disposable scalpel blade (Gillette Surgical, Great West Road, Isleworth, Middlesex). The loosened silica was drawn by suction (AEI Motor & Control Division, Newcastle, Staffs.) through a vacuum thimble filter. These filters consist of a sintered glass filter disc (approximately 10 mm diameter) encased in, and at one end of, glass tubing (40mm) to which at each end narrower tubing (3 x 20mm) is fused. These devices are a simplified version of the "vacuum cleaners" described by Matthews et al.(1962). The thimbles were then inverted and the material eluted by filtration through the sintered glass base of the thimble (Figure 4). Methanol: ammonium hydroxide solution (3 x 2ml.) was used for elution. The eluate was dried (in a water bath at 40°C) under a stream of dry oxygen-free nitrogen.

The purified labelled thyroxine was stored at -20°C in methanol: 0.5 molar ammonium hydroxide (2:1, v/v) at a concentration of approximately 20,000 d.p.m. per 50 ul. Working solutions were used within two weeks of preparation.

Liquid Scintillation Counting

Radioactivity was measured by liquid scintillation counting using a Nuclear Chocago Mark I Scintillation Computer (Nuclear Chicago Corporation, Inc. Des Plaines, Illinois, U.S.A.), calibrated for simultaneous counting of ^{14}C and ^{3}H employing three channels. The scintillation fluid was toluene based and contained PPO

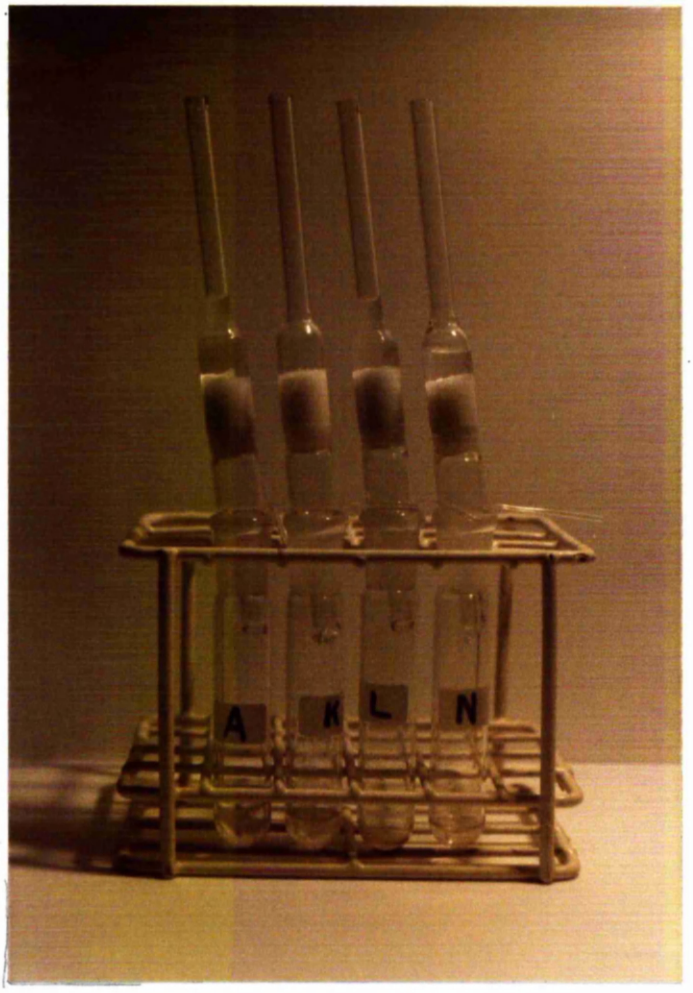


Figure 4 : Extraction thimbles used for the collection of silica from thin layer chromatography plates. Elution of iodoaminoacids from the silica was facilitated by percolation of solvent through the silica and sintered glass filter disc of the inverted thimble. The eluate was collected in test tubes.

(2,5-diphenyloxazole) (4g per litre) as the primary scintillator and POPOP (1,4-di(2-(5-phentloxozolyl)-benzene) (0.05g. per litre) as the secondary scintillator. Quenched standards (Nuclear Chicago Corporation Inc.) containing an identical scintillation solution were used to establish quench correction curves employing external standard channels ratio techniques. The external standard of ^{133}Ba is incorporated into the scintillation counter. Samples for assay were contained in scintillation fluid (20ml.) and counted for 20 minutes. Aqueous samples containing radioactively labelled thyroxine were blown to dryness under nitrogen at 40°C, and then redissolved in the scintillation fluid. All results of such liquid scintillation counting are expressed as disintegration per minute (d.p.m.) taking account of the counting efficiency of the apparatus as calculated by channels ratio.

Experimental Procedures

The procedures to be considered in this work are those designed to extract and derivatise urinary thyroid hormones so that quantitation by gas liquid chromatography would be possible.

Extraction of Urinary Thyroid Hormones

Urine is not applied directly to a gas liquid chromatography column. Substances in the urine are almost certain to interfere. An extraction step is unavoidable. This extraction procedure must permit the removal of proteins and other substances which might

interfere with the subsequent derivatisation, chromatographic separation and detection by electron capture detection. The use of the latter dictate stringent requirements for an extraction procedure. Although there are no described methods for extraction of the thyroid hormones from urine prior to gas liquid chromatography application, there are published preparative procedures for the quantitation of urinary thyroid hormones using competetive protein binding and radioimmunoassay methods.

Chan and her associates (Chan et al., 1972) has reported the application of a competitive protein-binding assay (CPB) for the estimation of thyroxine (T_4) in urine and radioimmunoassay (RIA) for the estimation of triiodothyronine (T_3) in urine. Both methods utilise extraction of one volume of urine (pH 4) with three volumes of ethyl acetate prior to the actual assay procedure. Black et al. (1973) using a different extraction procedure (cation exchange resin (Dowex 50) column), but the same assay method (competitive protein-binding) have found urinary thyroxine levels markedly lower than those of Chan. They suggest that the extraction procedures used by Chan produce hydrolysis of conjugates and they have questioned whether the method might not also promote partial deiodination of thyroxine to triiodothyronine. The values reported by Black et al. (1973) have been confirmed by other workers (Pastrana et al., 1975), who used RIA and unextracted urine. Also, cation exchange resin columns have been used successfully prior to gas liquid chromatographic quantitation of serum (Nihei et al., 1971).

Thus the method of cation exchange chromatography (Dowex 50 W-X2; hydrogen form) (Sterling et al., 1969 - Backer et al., 1967)

was worthy of trial for the extraction of urinary thyroid hormones prior to gas liquid chromatography

Cation Exchange Chromatography of Thyroid Hormones

The use of cross-linked polystyrene cation (strong-acid form) exchange resin for the separation of iodoaminoacids from biological fluid has been amply reported. This procedure has been applied to the extraction of thyroid hormones from serum (Baker et al., 1967; Sterling et al., 1969; Nihei et al., 1971; Tajuddin & Elfbaum, 1973), thyroglobulin digest (Sormichi & Ui, 1975; Ogawara et al., 1972; Rolland et al., 1970), serum dialysate (Petersen et al., 1976) and urine (Black et al., 1973). The sample is applied to the column in acid pH and the iodoaminoacids eluted separately by solvents of gradually increasing pH.

At acid pH, the iodoaminoacids being almost exclusively in their cationic form, are strongly held by the negatively charged groups ($-SO_3^-$) on the resin. As the pH increases, the ratio of zwitterions to cations increases, resulting in the rapid movement of the iodoaminoacids down the column (Figure 5). If changes in the pH of the eluting solution are made gradually in the region where the state of ionization of the iodoaminoacids undergoing separation is altered, then individual iodoaminoacids can be eluted separately. By careful selection of a precise pH, it is possible to control the relative rates of movement of the different iodoaminoacids. The nature of the iodoaminoacid side-chain markedly affects the arte of movement on the column. Increase in

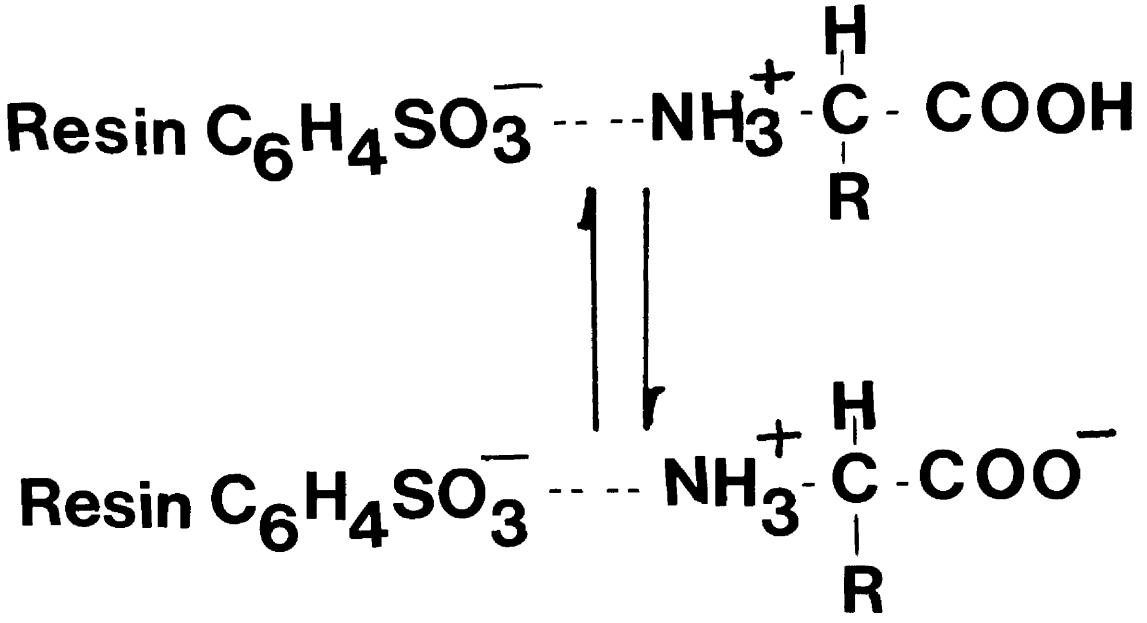


Figure 5 : Cation exchange between Dowex 50W-X2 (H^+) and iodoaminoacid at acid and alkaline medium. At acid pH, the iodoaminoacid being almost exclusively in their cationic forms, are strongly held by the negatively changed groups ($-SO_3^-$) on the resin. As the pH increases, the ratio of zwitterions to cations increases, resulting in the rapid movement of the iodoaminoacid down the column.

the size of hydrocarbon side-chain groups progressively reduces mobility. The presence of a hydroxyl group in the side-chain greatly increases the rate of elution, when chromatographed on a Dowex 50W column.

Alternatively all iodoaminoacids can be eluted simultaneously using either a mixture of concentrated ammonium hydroxide with water or alcohol (methanol or ethanol) at high pH.

Urine Collection and Storage

Urine collections (24-hours) were made in screw-capped opaque plastic bottles from patients in hospital. Each total urine volume was measured and the specimens were either processed immediately or deep-frozen until assay within 5 days of the collecting period. Prior to column chromatography, all urine samples were de-gassed by means of a vacuum pump, to avoid formation of air pockets which cause channelling of liquid or even complete cessation of flow through the column.

Volume of Resin and Urine Sample

The expected amount of thyroid hormones in complete 24-hour adult urine samples, by radioimmunoassay is approximately 1ug. of triiodothyronine and 1.5ug. of thyroxine (Black et al., 1975). There are no comparable values published for children. However I expected lower levels in children than those from adults because of a possible correlation with body weight (Hufner & Hesch, 1973). It

is clear that the volume of urine to be extracted should be large enough to yield sufficient hormone for final quantitation. Black et al. (1973) have extracted thyroxine from 10ml. adult urine and used 3ml. resin prior to their competitive binding assay method for the final quantitation. The competitive protein binding assay method for thyroxine in urine has been shown to give high values due to a contribution of urinary thyroid hormone metabolites and urinary triiodothyronine (Black et al., 1975). I have used 8ml. resin and 20ml. urine.

Preparation of Dowex 50W-X2 Resin

Standard grades of resin, as supplied, require a preliminary treatment to remove fine particles and soluble impurities (Gordon & Eastoe, 1964). The resin (10-20g.) was placed in an Erlenmeyer flask and well covered with deionized water. The resin was stirred to break up clumps of particles, so forming a suspension. This was allowed to stand overnight to permit full hydration (Sterling, 1971). The supernatant together with the "fines" (minute particles of resin still in suspension) was drawn off by using a water suction pump. The resin was again suspended in more water, allowed to settle, and the fines removed as before. The procedure was repeated (4 x) until the supernatant water was clear. Subsequently, the water was drawn off, and the resin covered with 5 molar hydrochloric acid. After shaking and settling the resin was washed with water until the water was neutral. This acid treatment was repeated thrice. The resin was then covered

with 1 molar sodium hydrxide which latter was removed after 4 hours. The resin was again washed several times with water until the water was neutral.

Preparation of the Cation Exchange Resin Column

Glass columns (Quick Fit CR12/10) were supplied by McFarlane Robson Ltd., Burnfield Avenue, Thornliebank, Glasgow. These consist of glass reservoirs (75ml. capacity) with their lower ends closed by a glass tap. The chromatographic glass column (120 x 10mm) has its lower end closed by a glass tap also and above this is fused a sintered glass plate (porosity 0) to support the column of resin. Columns were maintained vertical by clamping (Figure 6).

A 1:2 slurry of resin in deionised water (v/v) was maintained in aqueous suspension using a multispeed stirrer. The speed was adjusted to obtain a homogeneous suspension. The resin was then transferred to the column initially containing some water using an inverted pipette (10ml.). The excess water was allowed to drain from the bottom of the column as filling proceeded so that the water level was always above the surface of the resin. The resin was added to a height of 8 cm. Subsequently the resin was activated by washing with 1 molar hydrochloric acid (15ml.). This caused shrinkage of the resin. Then followed removal of the excess acid by rinsing with water (30ml.) until the water washings were neutral to litmus paper. This procedure caused the resin to swell again (Backer et al., 1967).

Figure 6 : Chromatographic column used in extraction of thyroid hormones from urine.

Determination of the Operating Capacity of the Column

The method described by Inczedy (1966) was followed. The volumes of urine to be extracted were chosen so that the whole sample was applied to the resin before the active hormones had appeared in the eluate fluid. Purified labelled thyroxine-2-^{14}C was used to monitor the behaviour of the thyroid hormones on the column.

To pooled urine from clinically euthyroid patients (60ml.) thyroxine-2-^{14}C (60,000 d.p.m.) was added. This urine was applied to the column at a constant rate (0.5ml. per minute) and the urine recollected in aliquots (5ml.). Each aliquot was blown to dryness in a water bath at 40°C under a stream of nitrogen and the radioactivity in each estimated. Breakthrough data were obtained according to the formula for each aliquot:

$$\frac{\text{d.p.m.'s in each aliquot of eluate fluid}}{\text{d.p.m.'s in similar aliquot of the original urine}} = \frac{C}{C_o}$$

Figure 7 shows the results obtained from a typical experiment. Since these data indicate that such columns can retain thyroxine in up to 30ml. urine, it was decided to use 20ml. urine in each experiment so as to be well within the capacity of the column.

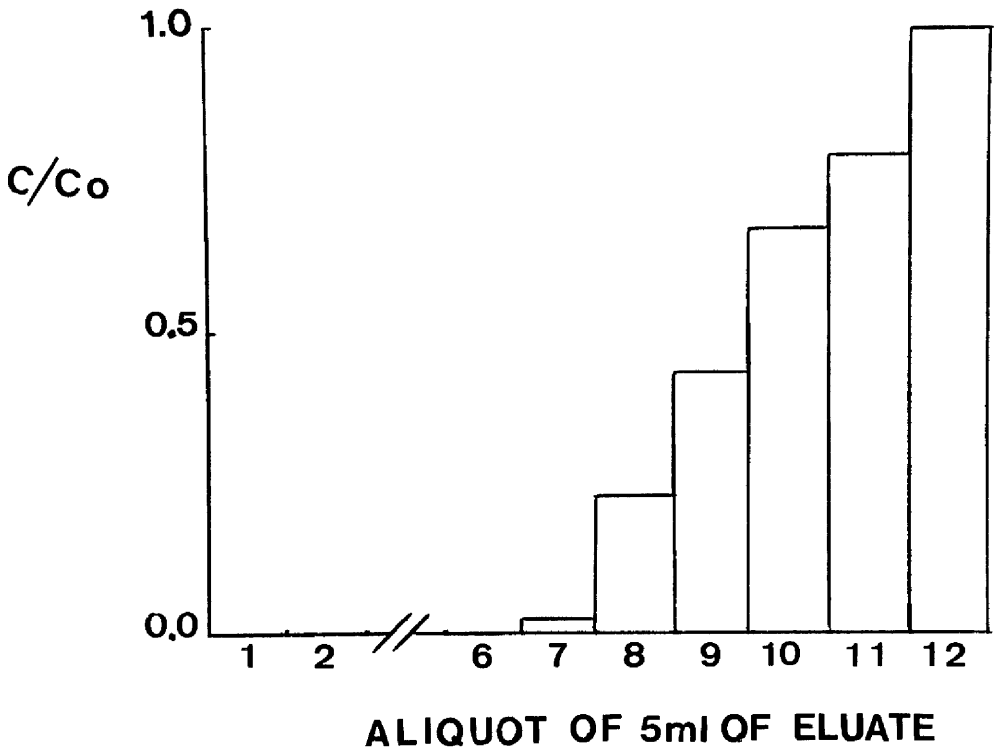


Figure 7 : Break through data to show maximum volume of urine applied to column before the compounds of interest appear in the eluate. The C/Co ratio being the ratio of d.p.m.'s in each 5ml. aliquot of urine from the column to the d.p.m.'s in 5ml. uncolumned urine. Breakthrough point is the seventh aliquot. Therefore the column can accommodate (operating capacity) up to 6 x 5ml. (30ml.) urine without leaking out hormone.

Determination of the Minimal Volume of Solvent Required for Elution of Thyroxine-2-^{14}C.

Urine (20ml.) was applied to the resin as before. Prior to elution, ammonium acetate (30ml.) (0.15 molar, pH 8.5) as suggested by Sterling et al. (1969) was chosen to wash through the resin bed. In addition the ammonium acetate is able to remove urinary proteins which may have been retained on the resin bed. Methanol:ammonium hydroxide (17 molar) (97:3, v/v); first used by Tajuddin & Elfbaum (1973) was used to elute the thyroid hormones from the column. Both solutions were applied to the column at a flow rate of 0.5ml. per minute. The first eluate (30ml.) was discarded and aliquots (2ml.) representing the methanol:ammonium hydroxide phase were collected. The radioactivity in each aliquot was assayed. The findings are shown in Figure 8.

It will be noted that exactly 95.403 per cent of the original radioactivity is contained in the first 14ml. of the column effluent collected. Thus a volume of 15ml. methanol:ammonium hydroxide was used for elution of thyroid hormones from the column. Thus approximately 95 per cent of the radioactivity and hence thyroxine applied to the column can be eluted. I have assumed that similar recovery of T_3 is possible.

Reproducibility of Column Extraction Method

Ten duplicate samples of urine (20ml.) were prepared as before but each pair contained varying amounts of thyroxine-2-^{14}C. Each

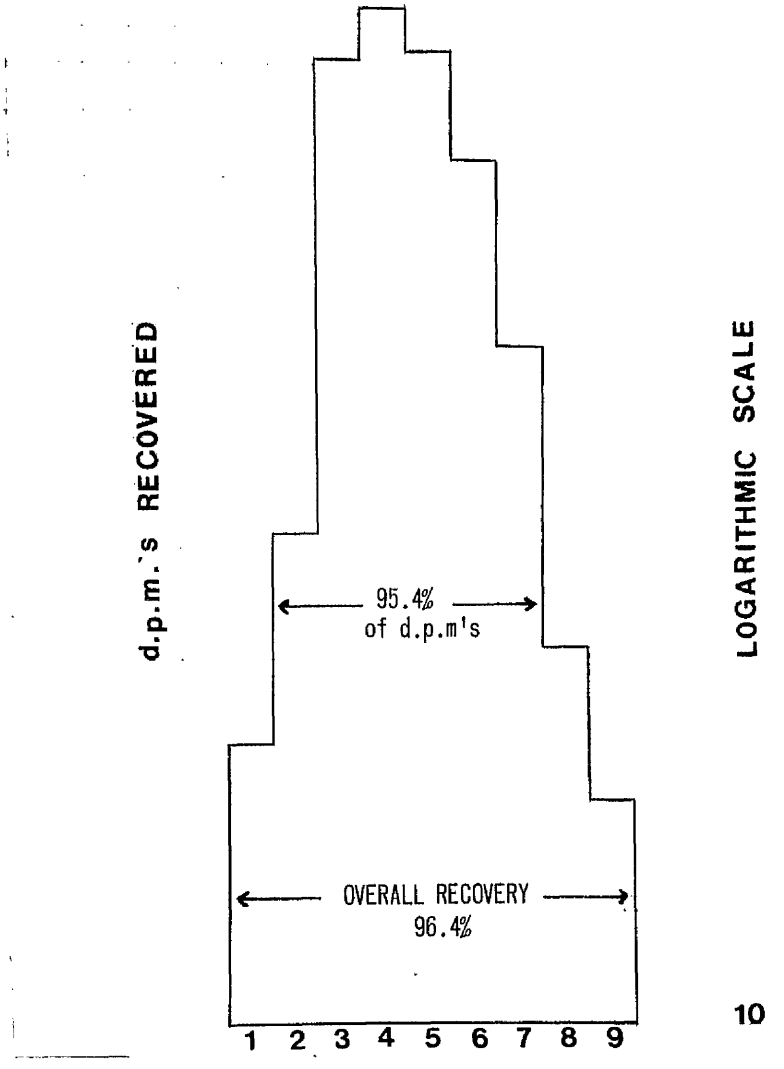


Figure 8 : Recovery of thyroxine-2-^{14}C from a Quick Fit CR12/10 column (12cm. x 1cm. I.D.) containing Dowex 50W-X2 (H^+) at 8cm. height. Urine (20ml.) containing 17,800 d.p.m. labelled thyroxine was percolated and washed by 30ml. ammonium acetate (0.15 molar, pH 8.5). Nine 2ml. aliquots of mobile phase (3 per cent ammonium hydroxide (17 molar) in methanol) were collected.

urine sample was processed on the column as described above and the recovery of radioactivity assessed in the methanol-ammonium hydroxide phase (15ml.). These results are shown in Table IX. It will be noted that good duplicate results were obtained and that the recovery rate was in excess, at all dilution, of 94 per cent.

Nature of Thyroid Hormones in Column Extract

Four column extracts of urine (4 x 20ml.) to which were added separately beforehand monoiodotyrosine (1ug.), diiodotyrosine (1ug.), diiodothyronine (1ug.), triiodothyronine (1ug.) and thyroxine (1ug.) were blown to dryness in a water bath at 40°C using a stream of dry nitrogen. The brownish residue was applied quantitatively to a thin layer chromatography plate. Standards were spotted on side lanes. The plate was then developed in the solvent system methyl acetate: isopropanol:5 molar ammonium hydroxide (45:35:20, v/v/v) (Chan & Landon, 1972) for 150 minutes (solvent front to 15cm.) (Figure 9). The R_f values of the standard thyroid hormones and their precursors are shown in Table X and Figure 10. It will be noted that in the thin layer chromatography system used complete separation of the iodothyronines is not achieved.

TABLE IX

Recovery rate of thyroxine-2-^{14}C from duplicate urine samples

After passage through Dowex 50W column using methanol/ammonium hydroxide (15ml.) as elutant

Original added d.p.m.	2224.21		1779.37		1334.52		889.68		444.84	
Eluate (14ml.) collected	Duplicate		Duplicate		Duplicate		Duplicate		Duplicate	
	a	b	a	b	a	b	a	b	a	b
dpm's in Eluate (15ml.)	2092.9	2137.4	1665.4	1680.9	1270.5	1243.4	844.2	858.2	440.3	437.6
% Recovery	94.1	96.1	93.6	94.47	95.2	93.18	94.9	96.5	99.01	98.4
Mean % Recovery	95.1%		94.03%		94.19%		95.7%		98.7%	

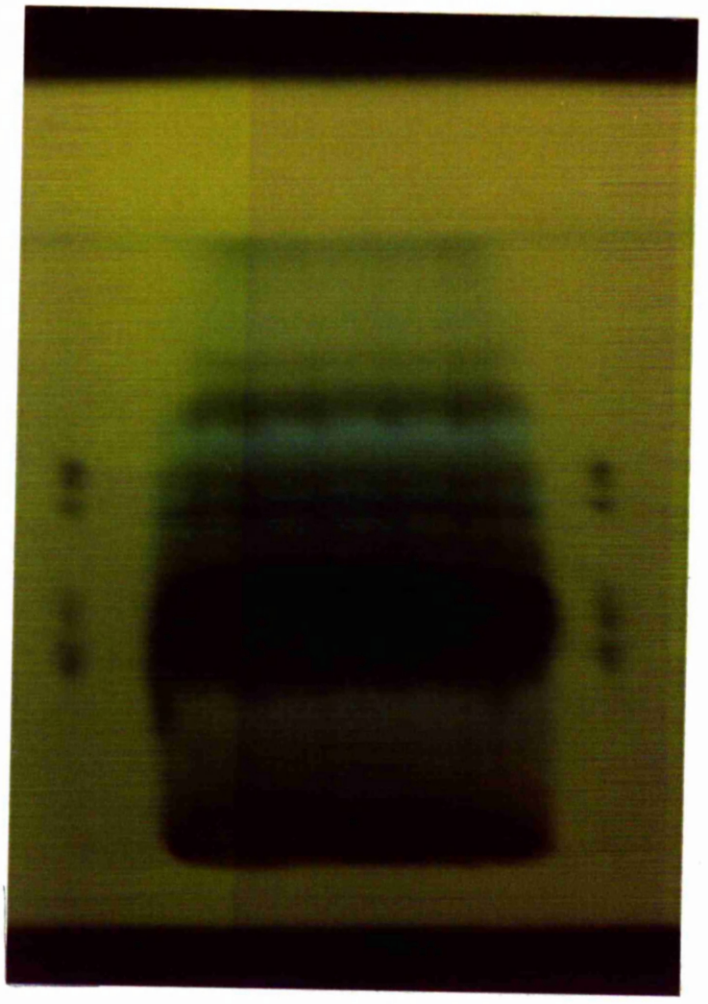


Figure 9 : One-dimentional thin layer chromatography of urine extracts (by column chromatography) in the solvent system: methyl acetate: isopropanol: 5 molar ammonium hydroxide (45:35:20, v/v/v) for 150 minutes using standards monoiodotyrosine(MIT) diiodotyrosine (DIT), diiodothyronine (T_2), triiodothyronine (T_3) and thyroxine (T_4) on both sides as markers. The plate was photographed under U.V. light.

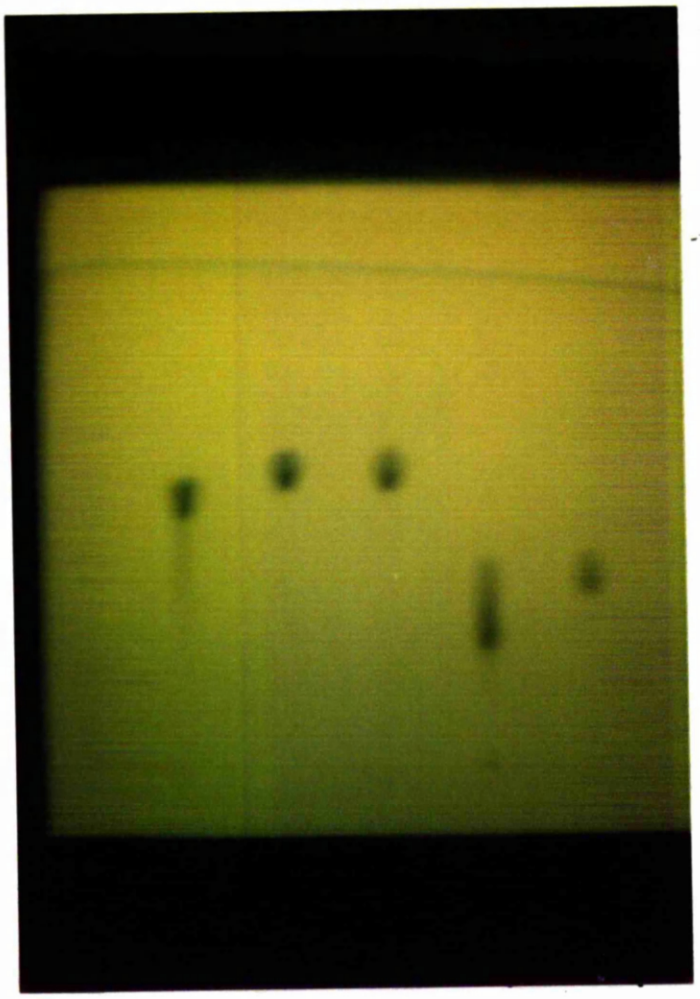


Figure 10 : One-dimentional thin layer chromatography of (from right to left) monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine standards in the solvent system: methyl acetate: isopropanol:5 molar ammonium hydroxide (45:35:20. v/v/v). Development for 150 minutes (solvent front 15cm.). The plate was photographed under U.V. light.

TABLE X : R_f values of standard monoiodotyrosine, diiodotyrosine diiodothyronine, triiodothyronine and thyroxine in the solvent system methyl acetate:isopropanol:5 molar ammonium hydroxide (45:35:20, v/v/v) on thin layer chromatography, developed for 150 minutes (Solvent front 15cm.).

Compound	R_f value	
	mean	range
Monoiodotyrosine	0.487	0.454 - 0.519
Diiodotyrosine	0.282	0.256 - 0.384
Diiodothyronine	0.585	0.525 - 0.683
Triiodothyronine	0.631	0.612 - 0.700
Thyroxine	0.598	0.539 - 0.656

Derivatisation of Urinary Thyroid Hormones

The thyroid hormones display a measurable volatility only at temperatures at which they decompose (thyroxine at 231°C). This resistance to volatility is due to the fact that thyroid hormones have large molecules (Table XI) and the molecules are mutually associated through polar groups (amino, hydroxy and carboxyl). Also these hormones are considerably labile and often decompose on contact with the reactive surface of a chromatographic support or with metals. Thus it is necessary to prepare suitable derivatives more volatile, less polar and suitable for gas liquid chromatographic separation. Marked enhancement of volatility and

TABLE XI : The molecular weights and melting points of monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine and their N,O-dipivalyl methyl esters.

Compound	As iodoaminoacid*		As N,O-dipivalyl methyl ester**	
	Mol. Wt.	M.p.	Mol. wt.	M.p.
Monoiodotyrosine	307	205°C	489	80°C
Diiodotyrosine	433	213°C	615	59°C
Diiodothyronine	525	256°C	707	93°C
Triiodothyronine	651.01	236°C	833	86°C
Thyroxine	776.9	231°C	959	103°C

* Merk Index (1968).

** Volpert et al., (1970).

suppression of the above undesirable effects can be achieved by effecting a derivatisation that blocks the possibility of intermolecular association and reduces the reactivity of the compounds.

For the analysis of thyroid hormones, which are known to have the highest molecular weight of all aminoacids, the choice of a convenient derivatisation procedure is narrowed considerably (see p.91 Discussion). Electron capture detection of N,O-dipivalyl methyl derivative (the only derivative adopted for serum analysis) allows nanogram (ng.) and picogram (pg.) quantities to be detected (Nihei et al., 1971).

I have used for the preparation of N,O-dipivalyl methyl derivatives iodoamino acid standards and urine extracts the method described by Hamilton (1973). In detail the procedure is as shown schematically (Figure 11). First, methylation of the carboxylic group with methanol in the presence of hydrochloric acid (as a catalyst). Second, Acylation of the other proton-carrying group (amino and hydroxyl) by reaction with excess of pivalic anhydride in the presence of triethylamine to bind the acid produced (pivalic acid).

One problem however is linked to these derivatives. Traces of acylation reagents (pivalic anhydride and triethylamine) and the so formed pivalic acid reduce the sensitivity of the electron capture detector (Nihei et al., 1971). Therefore a purification step utilising a thin layer chromatography system originally described by Stouffer et al., (1966) was used before actual chromatography.

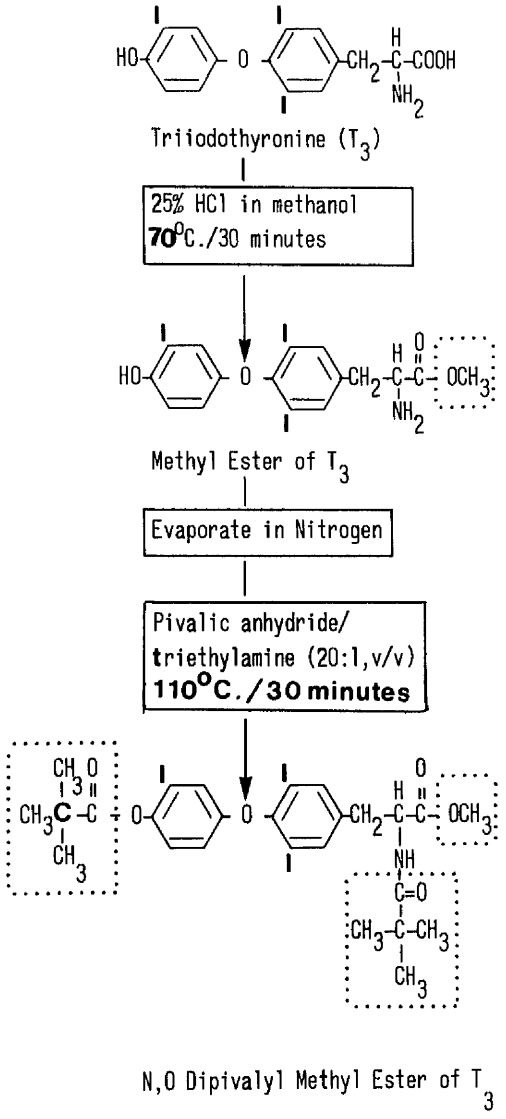


Figure 11 : Flow diagram of steps in the preparation of N,O-dipivalyl methyl ester derivative of triiodothyronine for gas liquid chromatography. First the terminal group is methylated to form methyl triiodothyronine which is then acylated with trimethyl acetic anhydride (pivalic anhydride) on the amino nitrogen and phenolic hydroxyl group to form the methyl N.O-dipivalyl derivative of triiodothyronine and an analogous derivative of thyroxine. The derivative chemical groupings are marked off with dots.

Preparation of Standard Derivatives

Authentic monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine (1mg. of each) was weighed accurately using a Cahn electrobalance (Model-10). The samples were placed in a separate acylation tube (5ml.) (Phase Separations Ltd.) (Figure 12). To the tube containing the thyroxine, thyroxine-2-^{14}C (20,000 d.p.m.) was added.

To each tube saturated hydrochloric acid-methanol (1ml.) was added and the tubes mechanically mixed (by Whirlimixer). They were then incubated at 70°C. in a thermostatically controlled oven (Hotbox model, Gallen Kamp) for 30 minutes. Subsequently the solution was evaporated to dryness under a stream of nitrogen.

To the dry residue dry methanol (20ul.) was added to aid solubilisation of the residue. Pivalic anhydride (0.2ml.) and triethylamine (10ul.) were then added. The tubes were closed after displacing the air with dry nitrogen. After mixing, the contents were heated for 30 minutes at 110°C to complete the derivatisation process, i.e. the formation of N,O-dipivalyl methyl ester. After cooling, the derivatives were evaporated to dryness in a sandbath at 40°C, using a stream of dry nitrogen. A thick brown oily residue was obtained.

Acylation of the methyl ester may occur at the amino or hydroxyl terminals (i.e. the N- or O-monopivalyl methyl ester) separately or concurrently to form the N,O-dipivalyl methyl ester. Quantitative conversion of the standard compounds to their N,O-dipivalyl derivatives was determined by applying the dried residue (taken up in dry methanol) to a thin layer chromatography

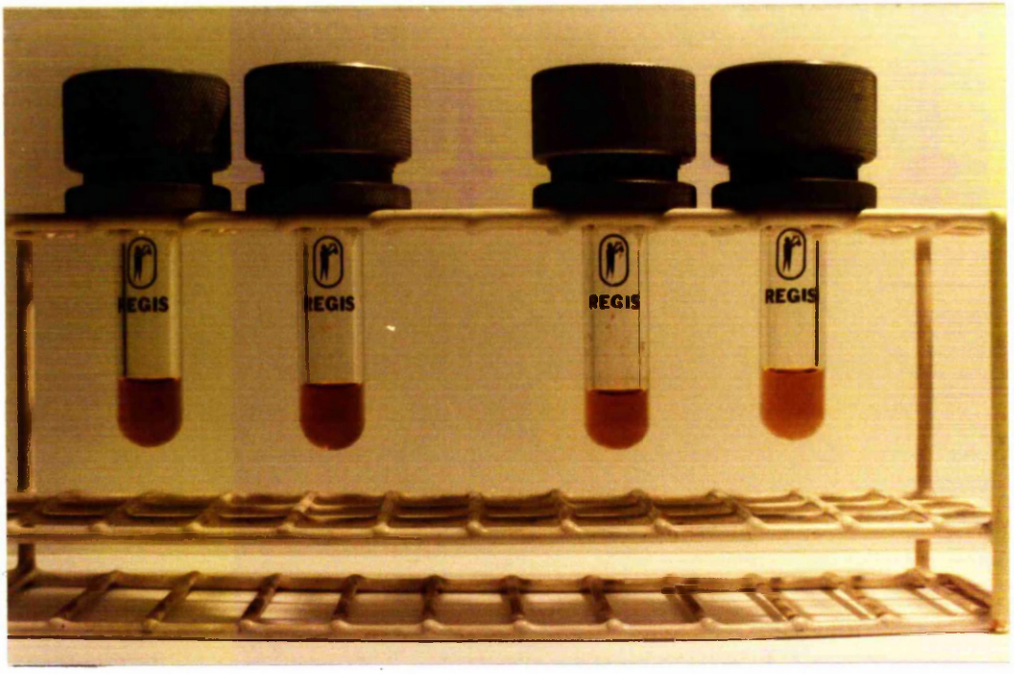


Figure 12 : Acylation tubes used to form the derivatives. These are heavy walled reaction tubes sealed by means of a two-piece threaded aluminium coupling. The top has an internal Teflon sealing disc.

plate. The plate was then developed in the solvent system chloroform:isooctane:formic acid (20:10:1, v/v/v) for 75 minutes. The lane containing the radioactive thyroxine was scanned (Figure 13). A single peak was found to correspond to the area of "cold" thyroxine N,O-dipivalyl methyl ester as seen under ultra violet light (Figure 14). The silica over the areas containing the respective dipivalyl methyl esters were removed and the purified derivatives eluted with methanol. By comparing the radioactivity of the ester with that originally added, an esterification rate of 99 per cent was achieved. It is assumed in this work that the conversion rate of the other compounds is of a similar order.

The N,O-dipivalyl methyl esters were redissolved in ethyl acetate to make a final dilution of 1ug. in 1.0ul. (for flame ionisation detection) and 10ng. in 1.0ul. (for electron capture detection) taking into account the efficiency of the derivatisation process. Gas liquid chromatography of standards esterified in this way were stable and they gave a single peak on the tracing (Figures 15 and 16). The standard solutions were stored in the dark at -4°C.

Derivativisation of Urine Extracts

It will be recalled that on page 53 column extracts of urine were purified by thin layer chromatography in the solvent system methyl acetate:isopropanol:5 molar ammonium hydroxide (45:35:20, v/v/v). The eluate from the thin layer plate was dried under a stream of nitrogen at 40°C for 20 minutes. The dried residue was derivatised as described above. After removing the excess reagents

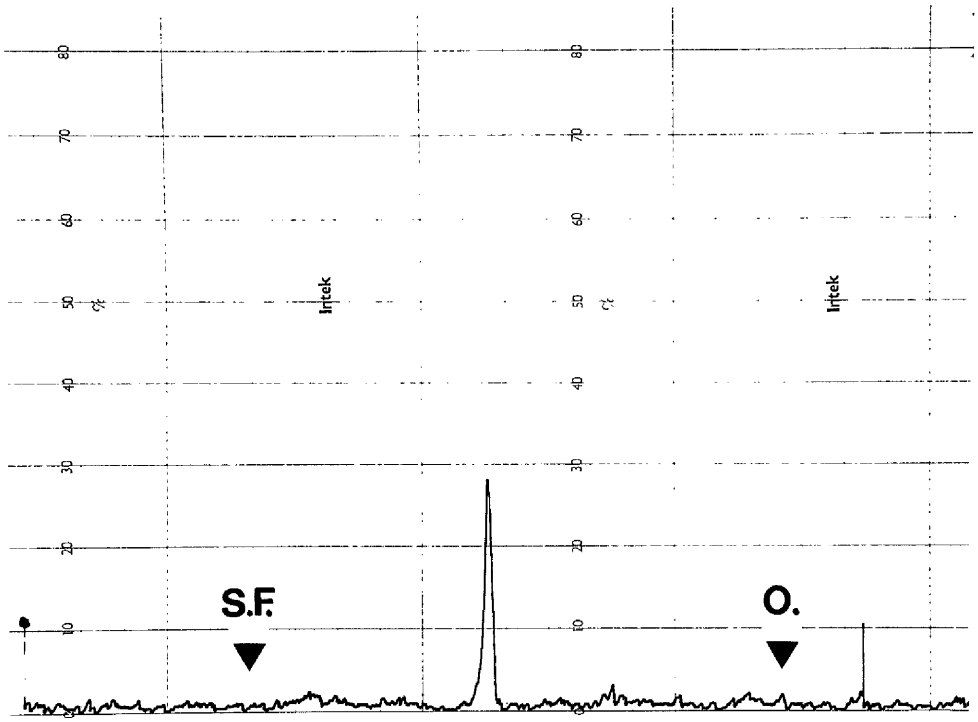


Figure 13 : Radiochromatogram scan of N,O-dipivalyl methyl ester of thyroxine-2-^{14}C on thin layer plate using Panax Radiochromatogram Scan (range 3 c.p.s., time constant 100 seconds and speed 30mm. per hour) after development in the solvent system; chloroform:isooctane:formic acid (20:10:1, v/v/v) for 75 minutes. The single peak corresponds to the area of cold thyroxine N,O-dipivalyl methyl ester when viewed under ultra violet light (Figure 14).

Figure 14 : One-dimentional thin layer chromatography of (from right to left) N,O-dipivalyl methyl esters of monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine standards in the solvent system; chloroform:isooctane:formic acid (20:10:1, v/v/v) for 75 minutes, as seen under ultra violet light.

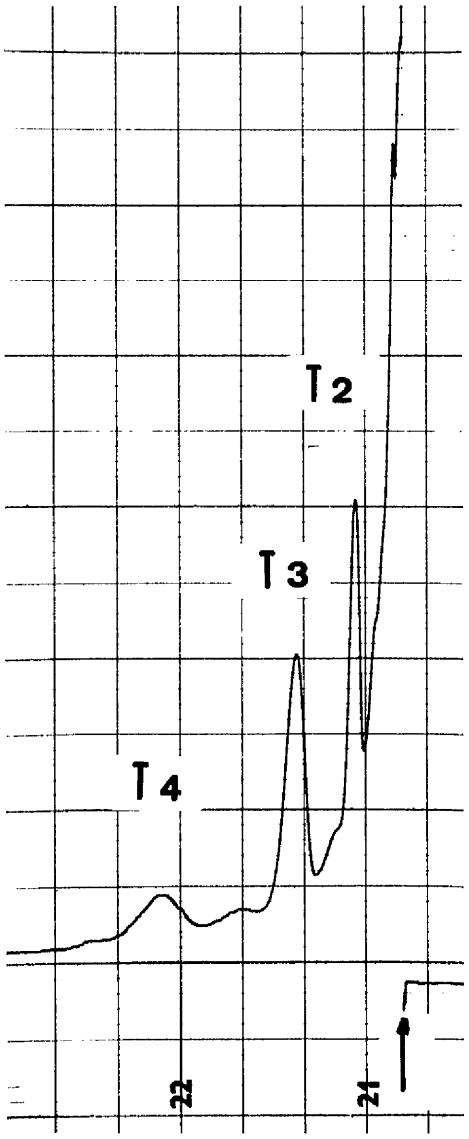


Figure 15 : Tracing obtained from isothermal gas liquid chromatography of standard N,O-dipivalyl methyl esters of diiodothyronine (T_2), triiodothyronine T_3 and thyroxine (T_4) (after purification by thin layer chromatography).

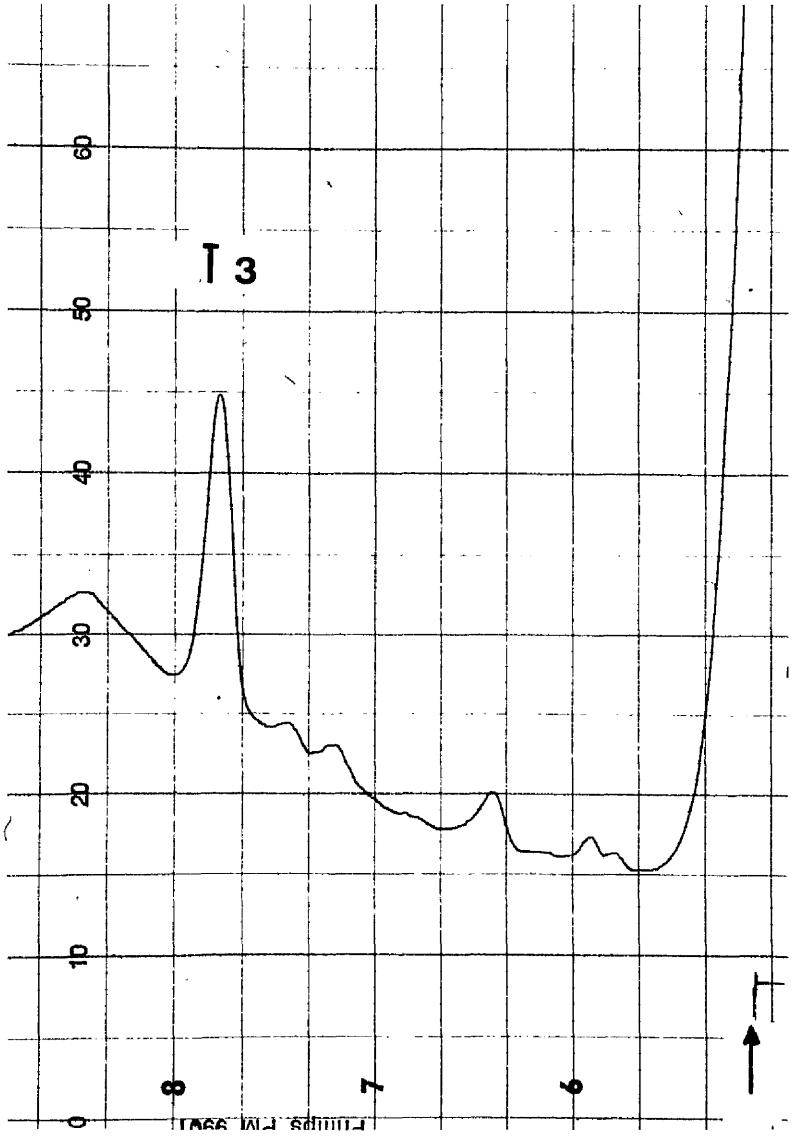


Figure 16 : Tracing obtained on gas liquid chromatography of standard N,O-dipivalyl methyl esters of triiodothyronine using temperature programmed conditions.

under nitrogen, the derivatised residue was dissolved in 50ul. of ethyl acetate and a 5ul. volume was injected into the gas liquid chromatography system. Preliminary experience indicated (Figure 17) that further purification of the derivatives was required before gas liquid chromatography-electron capture detection was attempted. This purification was achieved by thin layer chromatography in the solvent system chloroform:isooctane:formic acid (20:10:1, v/v/v) utilising purified standard esters as markers (Figure 18). The R_f values of the standard N,O-dipivalyl methyl esters of the thyroid hormones and their precursors are shown in Table XII.

TABLE XII : R_f values of standard monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine, N,O-dipivalyl methyl esters on thin layer chromatography developed in chloroform:isooctane:formic acid (20:10:1, v/v/v) for 75 minutes (solvent front 15cm.).

Compound As N,O-dipivalyl methyl ester	R_f
Monoiodotyrosine	0.29
Diiodotyrosine	0.32
Diiodothyronine	0.36
Triiodothyronine	0.45
Thyroxine	0.54

Although maximum and minimum R_f values are close, separation from unwanted material is satisfactory and subsequent gas liquid chromatography separation of the individual hormone esters is good (Figure 18).

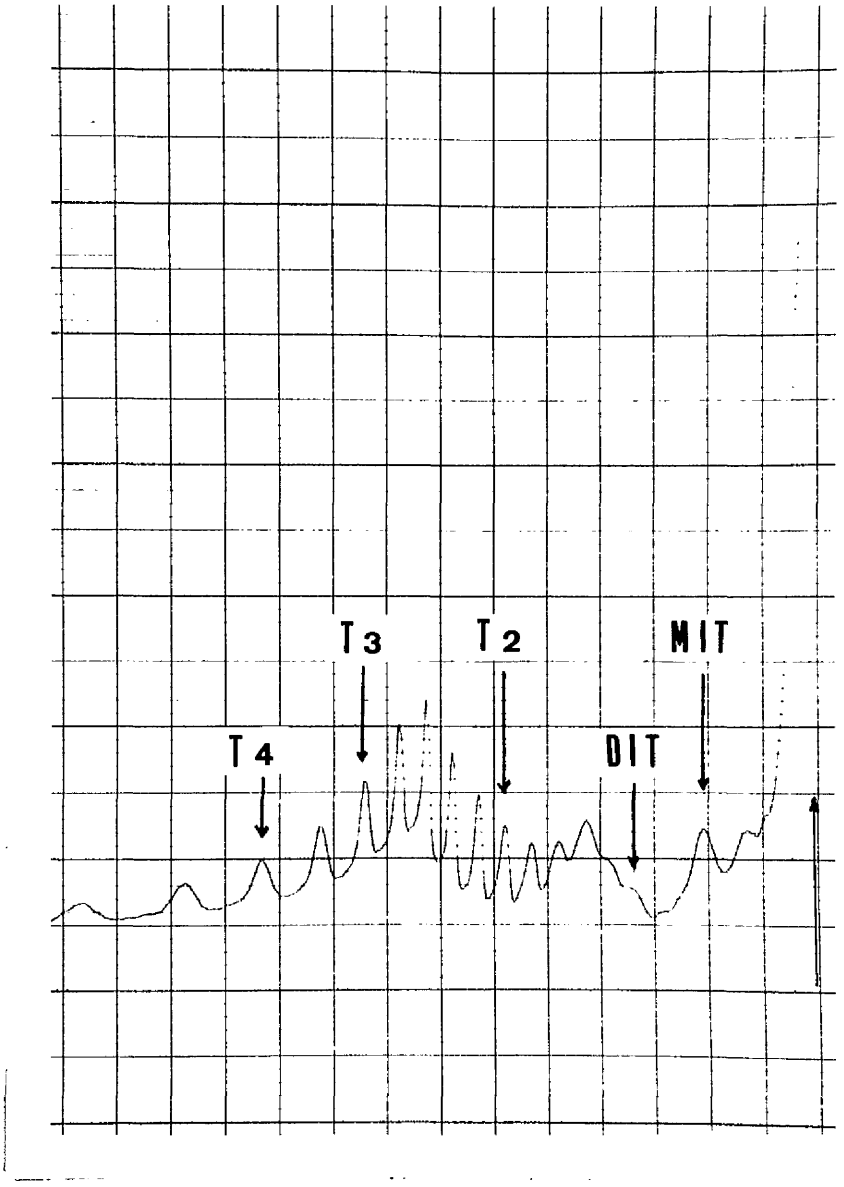
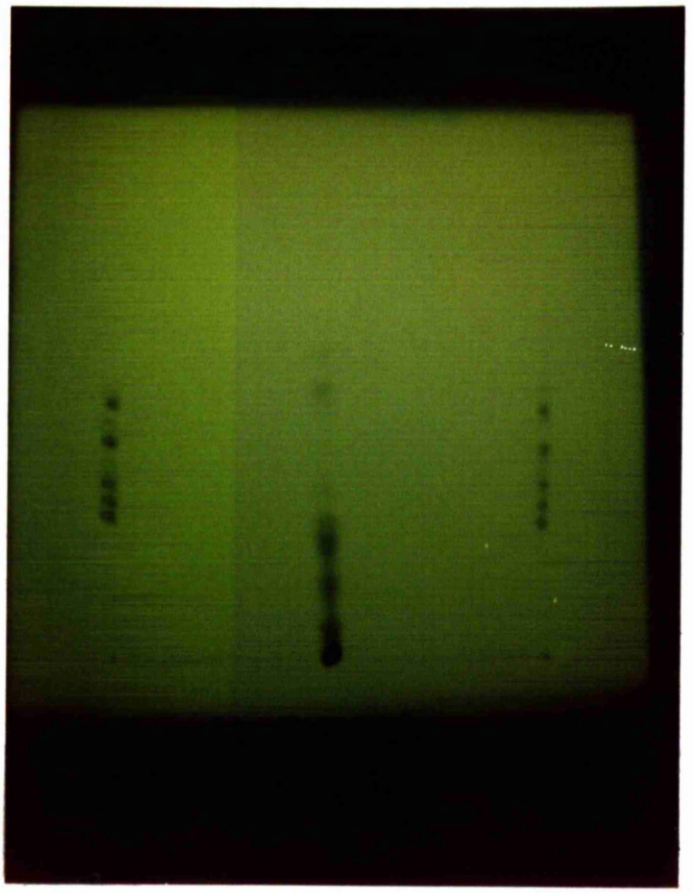


Figure 17 : Gas liquid chromatographic (FID) tracing of derivatised thyroid hormones extracted from urine. This many peaks indicates that the crude esters require further purification before proceeding to electron capture detection quantitation.



(5)
(4)
(3)
(2)
(1)

non-hormonal material causing peaks in tracing Figure 17.

Figure 18 : One-dimentional thin layer chromatography of derivatised urine extracts in the solvent system: chloroform: isooctane:formic acid (20:10:1, v/v/v) for 75 minutes using standard esters of monoiodotyrosine (1), diiodotyrosine (2), diiodothyronine (3), triiodothyronine (4) and thyroxine (5) on both sides as markers. The plate was photographed under ultra violet light. The test samples are seen more faintly in the centre lane.

Gas Liquid Chromatography

Preparation of Gas Liquid Chromatography Column

Only a few liquid phases fulfil the stability requirements for high temperature analysis of the diptivalyl esters of thyroid hormones. These temperatures range from 250-300°C. The stationary phases used by other workers were OV-1, OV-17, SE-30 and Dexil-300. The linear polyorganosiloxane OV-17 (methylphenyl organic groups) used by Hamilton (1973) possesses high chemical and thermal stabilities up to 350°C. However, the 2 per cent coating employed by the above author is not effective in deactivation of the support (Gas-Chrom Q) and it requires for full effectiveness silane treatment. This treatment reduces peak tailing to a considerable degree.

In order to reduce the adsorption properties of the support, two treatments are usually necessary. Firstly acid washing with strong mineral acid in order to remove metallic ions, and secondly deactivation of the support with a silanising agent, which converts the polar silanal groups into more inert silyl ether groups. An additional special treatment of the support by prolonged heating at 140°C for 4 hours at least gives the support the desirable feature of inertness. Such supports are usually identified commercially as "High Performance Grades". The type used in this investigation was Gas Chrome Q.

Coating the Support with Liquid Phase

The OV-17 solution was prepared by dissolving the OV-17 (1.5g.) in chloroform (100ml.), i.e. 1.5 per cent solution of OV-17, (wt/v). A 1.5 per cent solution of OV-17 applied to Gas Chrome Q (15g) gives a 3 per cent coating on the Gas Chrome Q, (100-120 mesh), (Gray, 1973).

The phase solution was applied to the support as described by Street (1969). The phase was introduced to the support under vacuum and the vacuum maintained. The mixture was gently agitated to dislodge bubbles. The vacuum was then released and the suspension allowed to stand at atmospheric pressure for a further 5 minutes. The slurry was transferred to a Buchner funnel and filtered under vacuum. Suction was discontinued after removal of excess solvent but before the solid residue in the funnel was dry. The coated support was then gently transferred to a watch glass (200mm) and allowed to dry in air for a few hours. The material was then dried in an oven at 100°-110°C for 1 hour. It was then ready for packing into a column.

Preliminary Silanisation of the Column

Prior to packing, the empty 5 feet stainless-steel column (4mm internal diameter) was rinsed with concentrated hydrochloric acid, washed with deionised water, silanised with 5 per cent (v/v) dimethyldichlorosilane in toluene for 15 minutes, and then rinsed with methanol and, after thorough drying at 100°-110°C for 1 hour,

the column was packed with the prepared coated support. If urine samples contain a very low concentration of thyroid hormones, silanisation of the inside wall of the column prior to packing will help minimize sample adsorption problems (Horning, 1968).

Packing and Conditioning the Column

The detector end of the column was connected to a water pump and gentle suction applied. The open end of the column was held upright and the coated support poured into the tube through a small filter funnel attached by clean rubber tubing. The column was tapped gently using a hand vibrator (Phase Separations Ltd.). A silanised glass wool plug was inserted into the injection end of the column and the column linked to the gas liquid chromatography apparatus.

The OV-17 liquid phase has a maximum operating temperature of 350°C. A slow flow (increasing from 10-40ml. per minute) of nitrogen carrier gas (oxygen-free) was maintained through the column as the temperature of the oven was raised slowly to 335°C. In this way excess phase "bled" from the column. Conditioning at this temperature was allowed to continue overnight before coupling the column to the detector. The optimum efficiency of a packed column is dependent upon the ratio of particle size and the bore of the column. The accepted particle size is 100-200 mesh with 4mm internal diameter columns (Pye Unicam Data).

Gas Liquid Chromatography Apparatus

The Pye Series : Model 85 heated electron capture/flame ionisation detector programmed gas chromatographic system obtained from Pye Unicam Ltd., York Street, Cambridge, England was used. Flame ionisation detection (FID) was used to determine the column separating powers in association with the following gas flow rates:-

oxygen-free nitrogen carrier gas - 40ml. per minute;

hydrogen - 40ml. per minute and air - 550ml. per minute.

All gases were supplied by the British Oxygen Company Ltd. Gas purifying bottles, packed with Molecular Seive 13 X (Pye Unicam Ltd.) were fitted in all gas supply lines, to remove traces of moisture and hydrocarbons.

Electron capture detection (ECD) using a 10mC ^{63}Ni source and a pulsating voltage of 150 uS was employed in the final separation and quantitation of extracts from urine.

The gas chromatographic curves were obtained on a 1 mv Philips flat bed recorder. Model PM 8000 (Pye Unicam Ltd.) operated at a chart speed of 2.5 mm per minute.

Operating Conditions of the Column

The separating powers of the column were determined by FID. Urine extracts were quantitatively analysed using ECD together with the recovery rates of radioactive material which had been added to the urine samples initially.

The 5 foot column prepared as described did not give a baseline separation of triiodothyronine dipivalyl methyl ester and thyroxine dipivalyl methyl ester (Figure 19). Such a column was replaced by a shorter one (3 foot), and the carrier gas (nitrogen) increased to 60ml. per minute. Resolution of these thyroid hormone derivatives was satisfactorily achieved using both an isothermal (290°C) and temperature programmed (220°-300°C) oven (Figure 20 and 21). Such columns were therefore used throughout the investigation. It will be noted that Figure 21 shows better separation of the various iodoaminoacids than does Figure 20. The oven temperature was programmed such that after 12 minutes of initial hold at 220°C, the temperature was allowed to increase at 2°C per minute from 220°C to 300°C with a final hold at 300°C for 5 minutes. The injection port temperature was maintained at 280°C and the electron capture detector at 350°C. Samples were injected onto the column using a Hamilton 10ul. syringe (Phase Separations Ltd.) fitted with an 11.5cm. needle.

An analytical tracing of normal human urine employing the above conditions is shown in Figure 22. The complete resolution of the triiodothyronine and thyroxine peaks facilitates quantitation. It will be noted that a number of peaks run ahead of the thyroid hormone derivatives. Among these peaks monoiodotyrosine, diiodotyrosine and diiodothyronine can be identified. The value of this finding will be discussed later on.

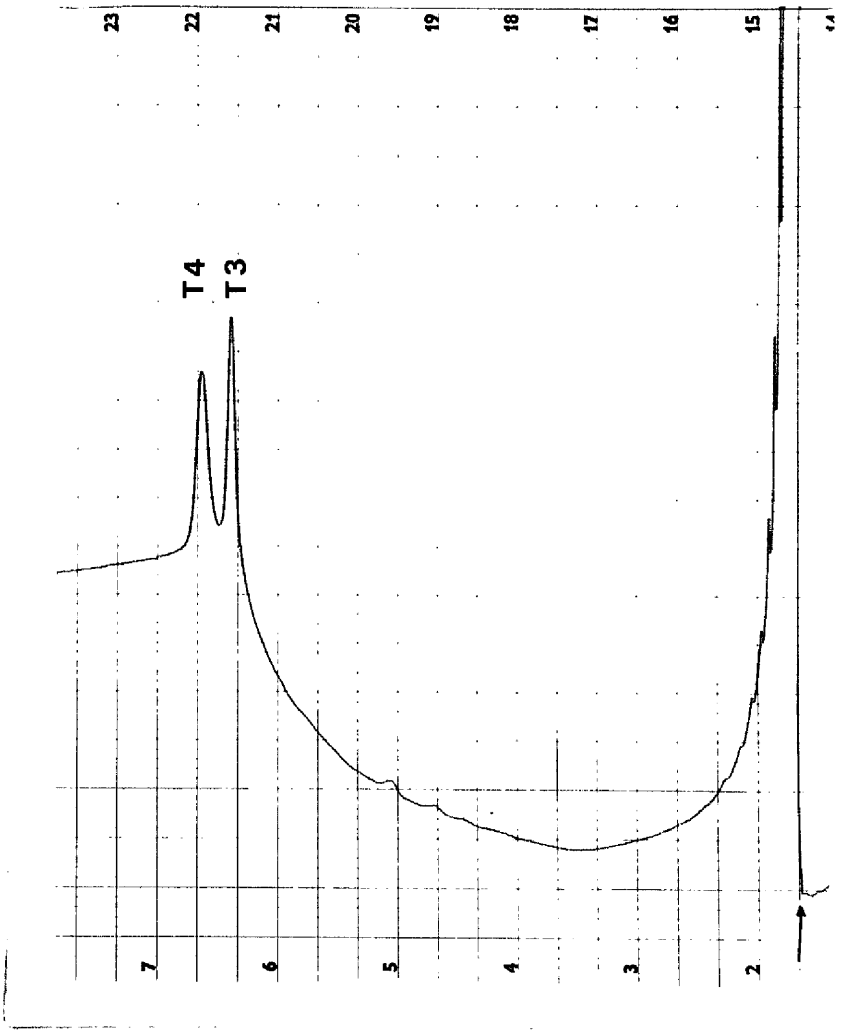


Figure 19 : Separation of the N,O-dipivalyl methyl derivatives of triiodothyronine (T_3) and thyroxine (T_4) using a 5 foot column; with 2 per cent OV-17 on Gas Chrome Q and the carrier gas at 40ml. per minute. Temperature programme is responsible for the base-line drift.

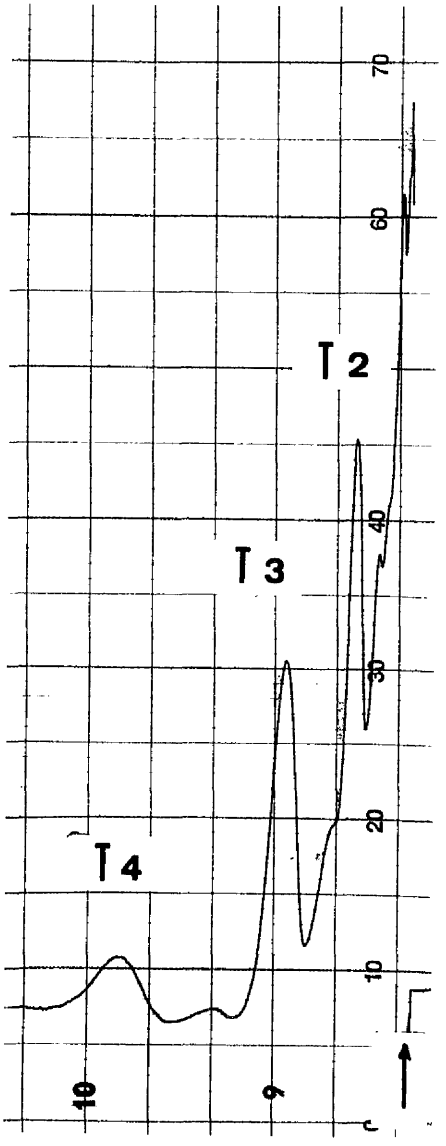


Figure 20 : Separation of the N,O-dipivalyl methyl derivatives of monoiodotyrosine (MIT), diiodotyrosine (DIT), diiodothyronine (T_2), triiodothyronine (T_3) and thyroxine (T_4) using a 3 foot column (3 per cent OV-17) and isothermal (290°C) operation. Note monoiodotyrosine and diiodotyrosine peaks are inseparable from the solvent front.

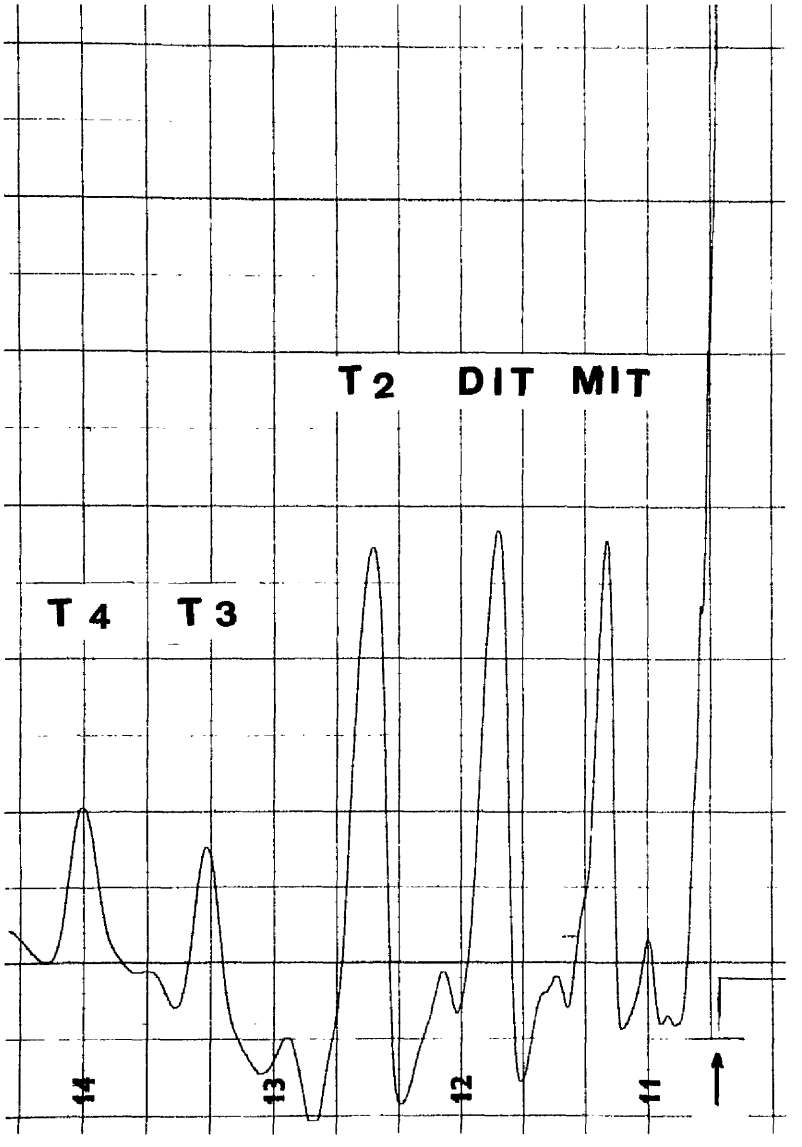


Figure 21 : Separation of the N,O-dipivalyl methyl derivatives of monoiodotyrosine (MIT), diiodotyrosine (DIT), diiodothyronine (T_2), triiodothyronine (T_3) and thyroxine (T_4) using a 3 foot column (3 per cent OV-17) and temperature programmed operation.

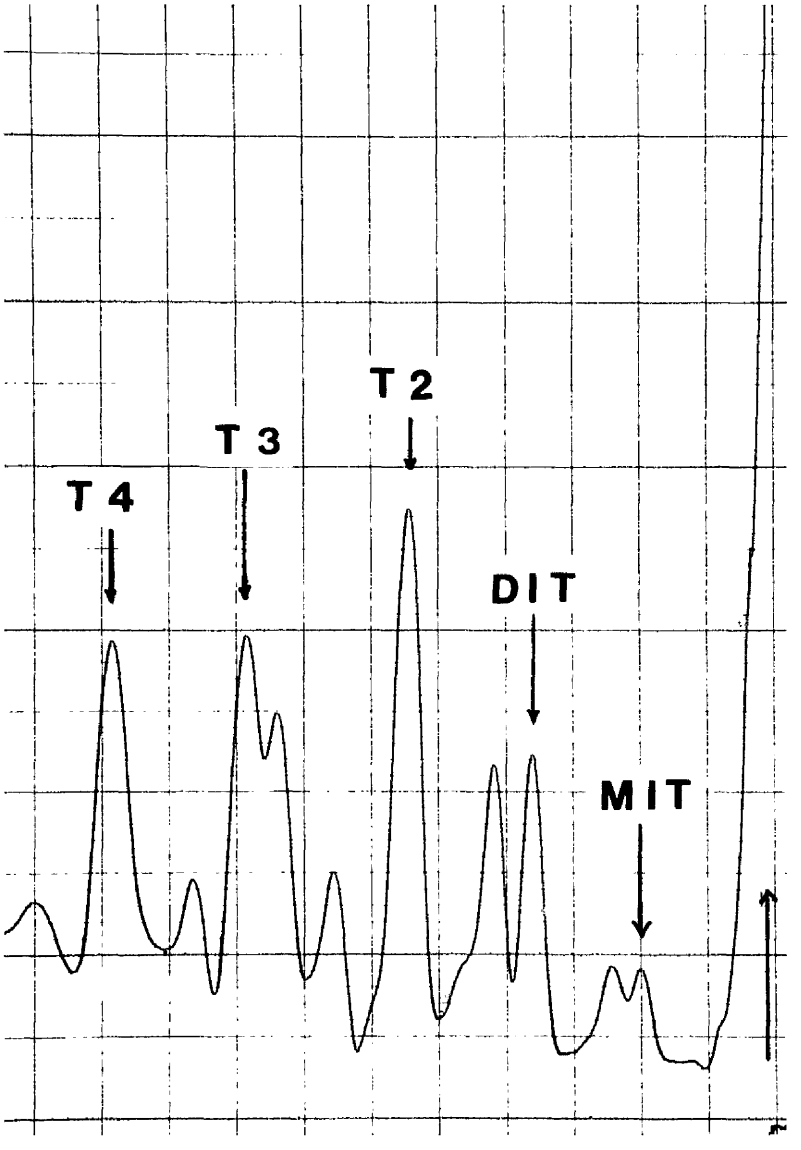


Figure 22 : A tracing of naturally occurring thyroid hormones from the author's urine (triiodothyronine (T_3) and thyroxine (T_4)).

Linearity of the Detector

The detector gave an almost linear response to thyroid hormone derivatives over the range required for analysis (Figures 23 and 24). Linearity of detector response to varying amounts of the thyroid hormone derivatives was determined.

a) by measuring the peak height alone.
b) by calculating the peak area determined from the width of the peak at 50 per cent of the height multiplied by the peak height.
c) by peak area determined by triangulation by halving the product of the peak width at the baseline multiplied by the peak height.

The linearity of detection using all three modes of quantitation is shown in Figure 25. Quantitation using peak height was chosen since fewer errors were likely to occur in the measurement of peak heights.

Quantitation of Thyroid Hormones in Urine

An attempt was made to employ diiodothyronine (T_2) for internal standardisation. The principle regarding the choice of an internal standard is that its retention time should be close to the sample component which is of interest (Pattison, 1969). The use of diiodothyronine was suggested by Volpert et al., (1970) and Docter & Hennemann (1971). A typical chromatogram of diiodothyronine and thyroid hormone derivatives is shown in Figures 20 and 21. Docter & Hennemann (1971) have found a constant ratio between the diiodothyronine peak and triiodothyronine and thyroxine peaks. However, the presence of diiodothyronine in urine samples (Figure 22)

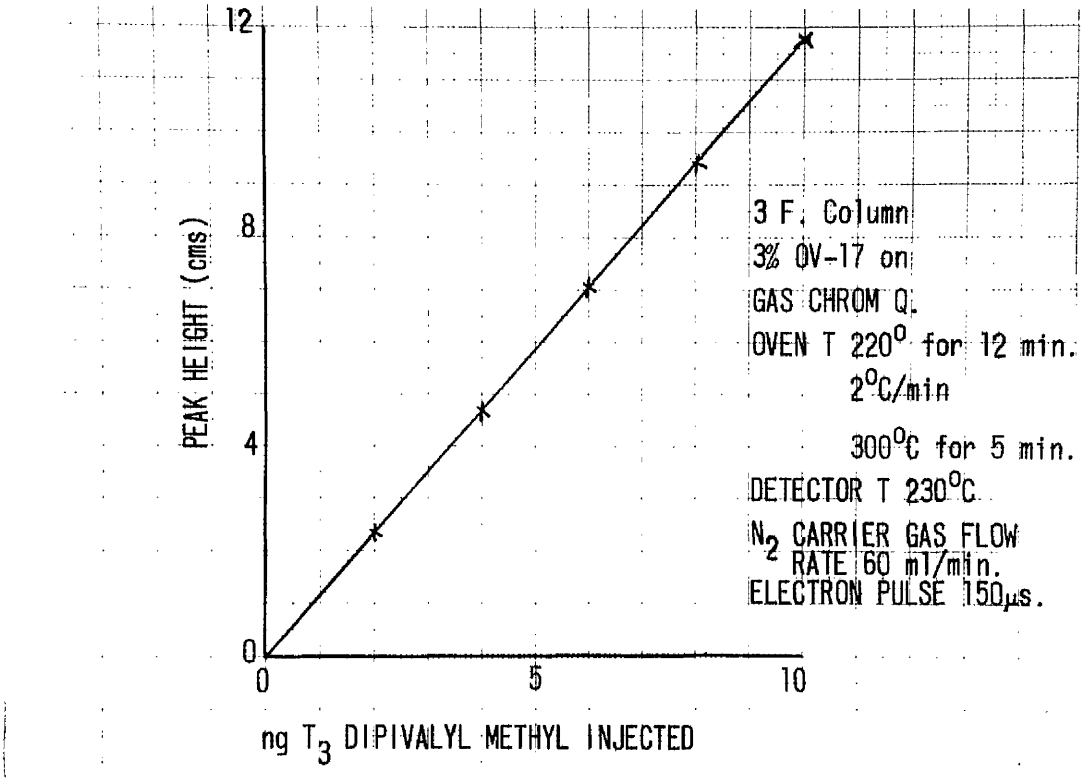


Figure 23 : Electron capture detector response (peak height) to varying amounts of methyl N,O-dipivalyl derivative of triiodothyronine.

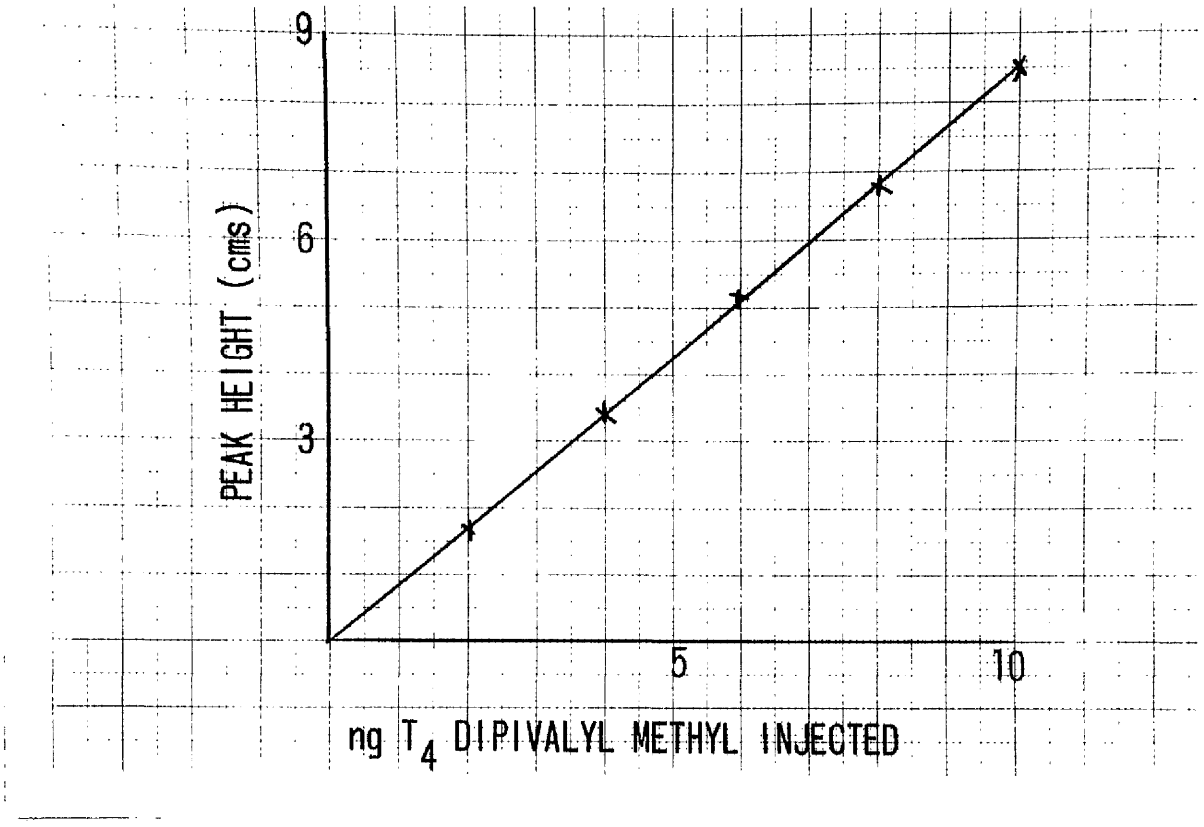


Figure 24 : Electron capture detector response (peak height) to varying amounts of methyl N,O-dipivalyl derivative of thyroxine. Operating conditions are the same as in Figure 23.

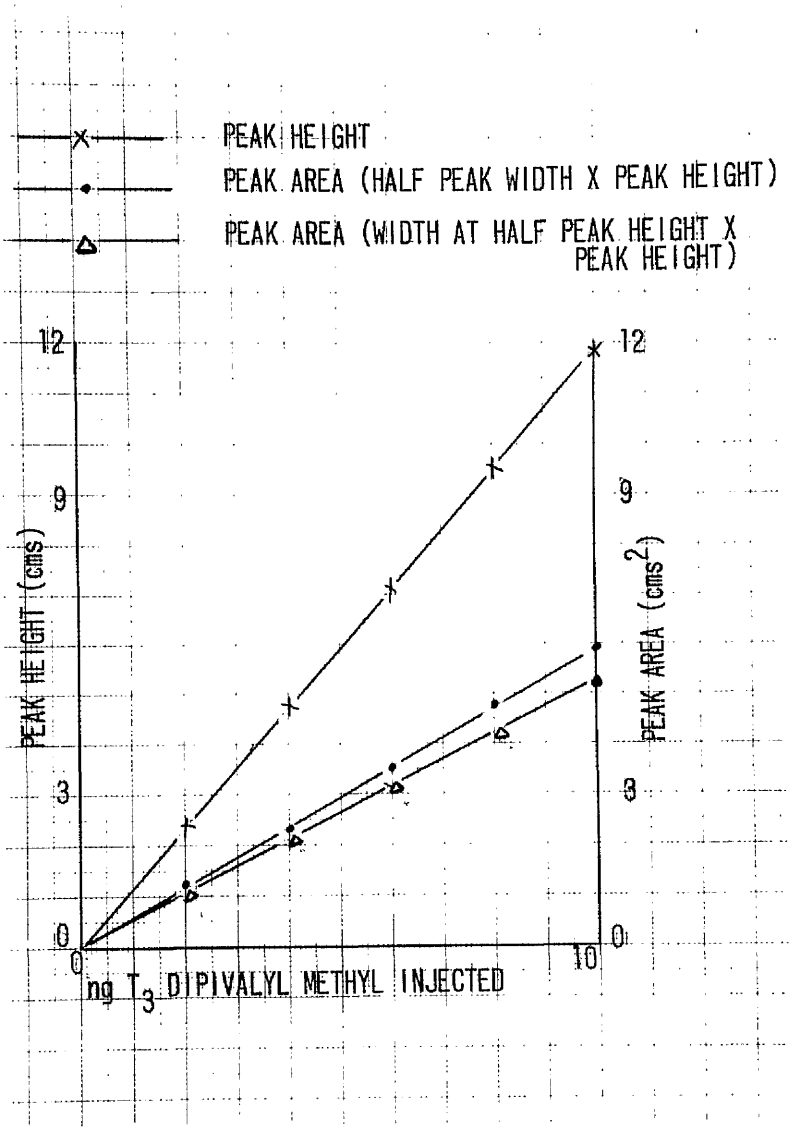


Figure 25 : Linearity of detector response (ECD) to dipivalyl methyl ester of triiodothyronine determined by measuring the peak height alone, by calculating the peak area determined from the width of the peak at 50 per cent of the height multiplied by the peak height, and by peak area determined by halving the product of the peak width at the baseline multiplied by the peak height.

precluded the use of this compound as an internal standard for quantitative studies. The external standardisation method was employed. Standard amounts of triiodothyronine and thyroxine N,O-dipivalyl methyl esters were injected prior to sample injection and unknown compared to known. It is of note that Nihei et al., (1971) have used this method for serum analysis.

Correction was made for the dilution of the final sample relative to the amount injected, for the total 24-hour urine $\frac{2}{3}$ volume, and for the percentage recovery as estimated by the recovery of thyroxine-2-^{14}C which had been added initially to samples.

Application of Methodology

In the foregoing the methodology has been shown to be efficient in the extraction, purification and gas liquid chromatographic separation of triiodothyronine, thyroxine and their precursors, and therefore the method was applied to urine samples from normal children and from patients with thyroid disorder. It proved to be able to detect the small amounts of these substances present in 20ml. urine. In outline the overall scheme is shown in Figure 26, and a step by step procedure is outlined.

Application of Experimental Procedures

(1) To urine (20ml.) a trace amount of purified radioactive thyroxine (approximately 20,000 d.p.m.) was added and the pH adjusted to pH 4 by the addition of hydrochloric acid.

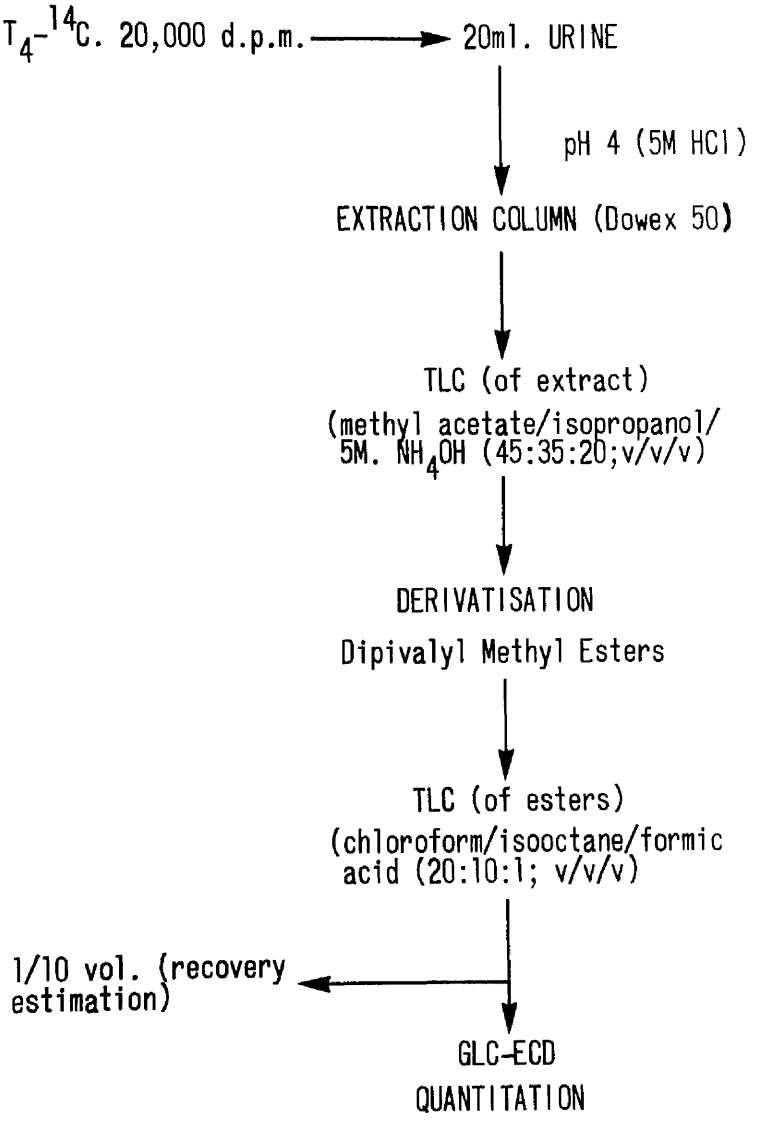


Figure 26 : Flow diagram of the procedures used in the estimation of urinary thyroid hormones.

(2) The urine was quantitatively percolated through the column (Dowex 50W) at 1-2ml. per minute. As the urine passed through the column a light brown area developed at the top of the column.

(3) Thereafter ammonium acetate (0.15 molar, pH 8.5) (30ml.) at a rate of 2ml. per minute and methanol/ammonium hydroxide (97:3, v/v) (15ml.) at a rate of 1ml. per minute were applied consecutively.

(4) The first 32ml. of the collected material from the column was rejected and the subsequent 12ml. collected.

(5) This latter was blown to dryness in a water bath at 40°C under a stream of nitrogen.

(6) The residue was dissolved in methanol/ammonium hydroxide (97:3, v/v) (60ul.) and 2ul. taken for recovery rate assessment (^{14}C counts).

(7) The remainder of the residue was applied quantitatively to a thin layer chromatography plate and authentic standards (monoiodotyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxine) spotted on side-lanes.

(8) The plate was developed in methyl acetate:isopropanol:5 molar ammonium hydroxide (45:35:20, v/v/v) for 150 minutes.

(9) After drying in air, the side-lanes were sprayed with ninhydrin and the area of silica corresponding to the area occupied by the standards removed and the silica eluted quantitatively with methanol (3 x 2ml.).

(10) The eluate was blown to dryness as before and the residue reconstituted in methanol (60ul.). An aliquot (5ul.) was taken for recovery estimation (^{14}C counts).

(11) The methanol was evaporated as before and residue placed in a dessicator for 24 hours.

(12) The dried residue was derivatised by the addition of methanol/hydrochloric acid (1ml.), the tube was securely stoppered and placed in an oven (70°C) for 30 minutes.

(13) The methanol/hydrochloric acid was removed under a stream of dry nitrogen; the tube was then placed in a sandbath and to the dried residue methanol (20ul.), pivalic anhydride (0.2ml.) and triethylamine (10ul.) was added.

(14) The tube was gently rotated to dissolve the residue thoroughly, stoppered and placed in an oven (110°C) for 30 minutes.

(15) The reaction mixture was taken to dryness as Stage 13.

(16) The residue was quantitatively applied to a thin layer chromatography plate and the plate was developed in the solvent system chloroform:isooctane:formic acid (20:10:1, v/v/v) for 75 minutes with standard derivatives on side-lanes.

(17) The area of the silica containing the derivatives was easily located under ultra violet light (254nm.). The silica from this area was removed and eluted with dry methanol (3 x 2ml.).

(18) The methanol was removed under a stream of nitrogen as at Stage 13, and the dried residue taken up in ethyl acetate (50ul.). An aliquot (5ul.) was taken for recovery assessment (^{14}C counts).

(19) Samples (10ul.) were injected into the gas liquid chromatography column, and the operation carried out under the conditions described in page 75.

(20) Following measurement of the respective peaks for sample and standards, the mass of hormone in a 24-hour urine sample was calculated taking account the dilutions and recovery rates at each step (Figure 27).

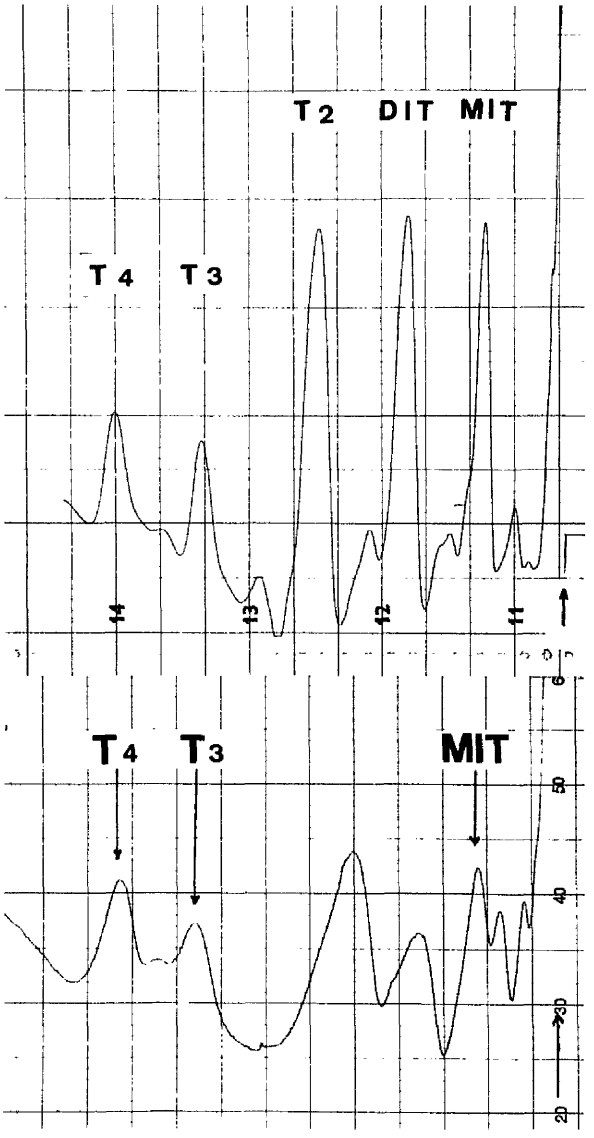


Figure 27 : Above; Standard trace

2ng. triiodothyronine methyl dipivalyl ester (T_3) gives a peak height of 3.3cm.

3ng. thyroxine methyl dipivalyl ester (T_4) gives a peak height of 2.5cm.

Below; Tracing of a urine extract from a euthyroid child

Arithmetic calculation for the peaks in Figure 27.

Thyroxine fraction:

Peak height of native ester = 2.7cm.

Peak height of 3ng. standard ester = 2.5cm.

Therefore by comparison of peak height ratios, thyroxine in sample injected (9.5ul. of 50ul.) = $\frac{2.7 \times 3}{2.5}$ ng.

and thyroxine in urine residue = $\frac{2.7 \times 3 \times 50}{2.5 \times 9.5}$ ng.

One-tenth of the residue contains 1,118.1 d.p.m.'s = 5.82 per cent of that added initially (19,193.7 d.p.m's). Thus if the recovery of the native thyroxine is equivalent to the recovery of thyroxine-2-^{14}C derivative from thyroxine-2-^{14}C added to the urine, then there is 58.2 per cent of native thyroxine present in the final residue.

Therefore in the aliquot processed (20ml.) there are = $\frac{2.7 \times 3 \times 50 \times 100}{2.5 \times 9.5 \times 58.2}$

The total 24-hour urine value = 320ml.

Therefore in 24-hour urine volume = $\frac{2.7 \times 3 \times 50 \times 100 \times 320}{2.5 \times 9.5 \times 58.2 \times 20}$ ng.

= 468.8ng. thyroxine are excreted per 24-hours.

Triiodothyronine fraction:

Peak height of native ester = 2.4cm.

Peak height of 2ng. standard ester = 3.3cm.

Therefore triiodothyronine in sample injected (9.5ul. of 50ul.) = $\frac{2.4 \times 2}{3.3}$ ng.

and triiodothyronine in urine residue = $\frac{2.4 \times 2 \times 50}{3.3 \times 9.5}$

and in aliquot processed (20ml.) = $\frac{2.4 \times 2 \times 50 \times 100}{3.3 \times 9.5 \times 58.2}$

and in 24-hour urine volume = $\frac{2.4 \times 2 \times 50 \times 100 \times 320}{3.3 \times 9.5 \times 58.2 \times 20}$

= 210.46ng. triiodothyronine are excreted per 24-hours.

Discussion of Methodology

As I have already noted the goal of this thesis was to develop an analytical methodology that would permit the simultaneous analysis of the unconjugated (free) thyroid hormones in urine. The expected amount of these substances in complete 24-hour urine samples was in the region of 1ug. triiodothyronine and 1-1.25ug. thyroxine (Black et al., 1975). The gas liquid chromatography methodology proved to be capable of detecting these small quantities. The method however cannot be used directly on crude urine as the urine contains a wide range of potentially volatile interfering compounds. A preliminary extraction step is unavoidable. This procedure should permit the removal of proteins and other substances which might interfere with the subsequent derivatisation, gas liquid chromatographic separation and detection by the electron capture detector. As the thyroid hormones tend to be adsorbed on to glassware (Lee & Pileggi, 1971), this adsorption could create errors in terms of accuracy and reproducibility of the method. Clean and inert glass surfaces were therefore ensured by silanization of the glassware with dimethyldichlorosilane.

Cation exchange chromatography has been shown to be a useful method for isolating thyroid hormones from biological fluids (Sterling et al., 1969; Nihei et al., 1971; Backer et al., 1967; Black et al., 1973). I have applied this method without modification except for quantity, to the extraction of these hormones from urine. The good recovery rate from the Dowex 50 column amply justifies the application. However because there was in the column effluent some

proteins to which the iodinated hormones might bind, it was necessary to introduce a "clean-up" step. A simple thin layer chromatography purification separated well the active thyroid hormones from unwanted material. Chan & Landon (1972) used this same thin layer chromatography system to confirm the presence of thyroxine in urine extracts. It will be noted that the system chosen isolates the "sought-after" hormones albeit closely related on thin layer chromatography but relatively free from undesirable contaminants.

One disadvantage of silica gel is that it usually contains a small amount of iron which may catalyse certain deiodinations and thus create artefacts. Such artefacts can be avoided by using acid-washed silica gel for the preparation of the plates (West et al., 1965). However when this step was attempted the silica gel crumbled and fell from the plates when alkaline and acid aqueous solvents were subsequently applied. No account therefore was taken of this theoretical deiodination.

Pure extracts of thyroid hormones are relatively thermolabile in their ionised form. It is thus necessary to prepare suiatble derivatives which are more volatile, less polar, and therefore suitable for gas chromatographic separation. The choice of a derivatisation procedure, for the analysis of thyroid hormones which have the highest molecular weights of all the amino-acids, is very limited. There are two possibilities:-

(1) Silylation of the amino-acid with bis (trimethylsilyl) acetamide or condensation with prehalogenated acetone, and

(2) Acylation of their methyl esters with trifluoroacetic acid anhydride, or trimethylacetic anhydride (pivalic anhydride).

Funakoshi and Cahnmann (1969) have shown that trimethylsilyl derivatives (TMS) of iodoaminoacids are partially destroyed on the column when injected in nanogram amounts. Additionally trimethylsilyl esters are readily hydrolysable by traces of moisture.

A great advantage of the oxazo-derivatives of monoiodotyrosine and diiodotyrosine is their stability, easy preparation and high responsiveness to electron capture detection (Husek, 1974; Husek & Macek, 1975). However Husek found great difficulty in eluting the derivatised thyroid hormones from the gas liquid chromatography column (an exceedingly long retention time is assumed to be the meaning of his statement).

Trifluoracetylation of thyroid hormones is easily achieved and the derivatives (TFA) are readily detectable by flame ionisation detection (Richards & Mason, 1966). However these derivatives are reported (Docter and Hennemann, 1971) to break down when injected in amounts suitable for detection by electron capture, such as might have been expected in physiological samples.

The pivalyl methyl esters are however the ideal derivatives of the thyroid hormones for gas liquid chromatography handling because they are not readily broken down either by traces of moisture or by oven temperature. Indeed they are less volatile than the other derivatives already discussed and therefore they volatilise at higher temperatures. I therefore chose pivalyl methyl esters of urinary thyroid hormones as the derivatives most likely to give reasonable results although their preparation is relatively more rigorous. Additionally the prepared esters required further purification

before injection because the reaction reagents (pivalic anhydride and triethylamine) and the so formed pivalic acid have a high boiling point and therefore without purification contamination of the column and interference with quantitation would have resulted. This might be regarded as a drawback as it is not shared with other derivatives. The purification step was that described by Stouffer et al., (1966) being a simple thin layer chromatographic system. Nihei et al., (1971) used for a corresponding step anion exchange column (Amberlit IR45). In my own experience my esters were not stable on such a column.

Finally on the problems of methodology, despite the various purification steps introduced, a large number of peaks were obtained from test samples of urine. These peaks clearly represented derivatives of other amino-acids but it was found possible to cause these to run ahead of the thyroid hormone derivatives by using a temperature programmed procedure rather than isothermal conditions. Among these peaks can be identified mono-iodotyrosine, diiodotyrosine, diiodothyronine, and therefore this should greatly facilitate future studies designed to elucidate thyroid hormone metabolism.

Organic iodine compounds in urine represent about 10 per cent of the thyroid hormones metabolised (Pittman et al., 1972). Most of them are not yet known. Those identified represent the main three routes of thyroid hormone metabilsm, viz. deiodoination, conjugation and side chain metabolism. Mono-iodotyrosine and diiodotyrosine have been identified as deiodinated urine metabolites of triiodothyronine and thyroxine (Roche et al., (1952 (a); 1952(b)), and diiodothyronine as the major metabolite of triiodothyronine

(Folk et al., 1960). Complete deiodination of thyroid hormones may also occur, leaving only the thyronine nucleus (Pittman et al., 1972). Burke et al., (1972) found 0.65 and 3.69ug. per day respectively of triiodothyronine and thyroxine conjugates in the urine. Oxidative deaminationand decarboxylation of the alanine side-chain to their acetic acid derivatives is the predominant metabolic pathway for thyroid hormones in the kidney (Albright et al., 1959; Etting & Barker, 1959; Pittman et al., 1972). Tetra- and tri-iodothyroacetic acid and pyruvic and lactic acid derivatives of thyroxine and triiodothyronine have been demonstrated in urine. The average daily excretion of thyroacetic acid in urine, from a thyroxine origin, is approximately 7.5ug. per day (Pittman et al., 1972). Administered thyroxine may also appear in urine as tetraiodothyropropionic acid (Roche et al., 1954). Thus some of the peaks unidentified could represent some of these compounds.

The possible coupling of gas chromatography to mass spectrometry is unique in that the structure of unknown compounds and their quantitation at femtomole levels is possible, and could be a part of a more advanced application of this work. The mass spectrometer has already been used in the study of standard N,O-dipivalyl methyl ester of diiodothyronine (Stouffer et al., 1966) and the value of the dipivalyl derivatives of thyroid hormones and some of their derivatives in hepatic hydrolysates and bile is under active investigation (Hoffenberg et al., 1974). It is clear that studies in this direction on urine samples, utilising the method developed here might help in the understanding of thyroid hormone metabolism, and excretion of metabolites. It is quite clear that it would be

possible to link gas chromatography-mass spectrometry with gradient elution to isolate individual iodinated urinary metabolites of the active thyroid hormones and so to reach in abnormal cases, blocks of synthetic or metabolic pathways. Such studies have to rely on paper chromatography of butanol extracts of serum or urine following administration of labelled thyroid hormones or their precursors (Mosier, 1975). The necessity for relatively large doses of radio-iodine in order to ensure sufficient radioactivity for measurements on the resultant chromatograms tends to discourage studies with these techniques especially in the paediatric age groups. Finally by urine analysis in athyreotic patients under treatment it would be possible to study the metabolism of exogenous triiodothyronine and thyroxine.

In conclusion, the analysis of the free thyroid hormones in urine presents a major analytical challenge. Not only is the composition of urine itself extremely complex, but the free hormones are present along with a variable amount of metabolites most of which are not identifiable. Further, the large molecular weight of these thyroid hormones (approximately 1,000), the lability of their iodine atoms, their tendency to adsorb on solid surfaces, and their presence and availability in only trace amounts in urine (in the free form), were the problems which had to be overcome in the present work.

CHAPTER 3

RESULTS WITH DISCUSSION

Introduction

Since the earliest report (Chan & Landon, 1972) on the measurement of urinary thyroid hormones, no agreement on significant levels has been reached. Indeed neither is it agreed that the urinary hormones represent the free ultrafiltrable serum thyroid hormones. Thus the clinical value of any results obtained is still sub judice. I present in this thesis, my own values of thyroid hormones in urine found in a group of euthyroid children and I present also some corresponding data from other children with thyroid disease. These patients were mainly in the Royal Hospital for Sick Children, Yorkhill, Glasgow.

Patients under Study

The euthyroid group consisted of 50 in-patients who had non-endocrine medical or surgical conditions and significantly they were without renal or hepatic disease. The patients investigated are listed in Table XIII. An identification number, age and clinical status is also shown. Those patients having treatment with drugs likely to affect the efficiency of thyroid binding globulin (phenytoin and salicylates) or its circulating level (glucocorticosteroids) are indicated thus (*). The male

TABLE XIII : Data on the euthyroid subjects used as normals in this investigation

MALE				FEMALE			
Patient	Patient Number	Age Years	Clinical Diagnosis	Patient	Patient Number	Age Years	Clinical Diagnosis
C.B.	1	3	Inguinal Hernia	T.M.	26	4	Fallot's Tetralogy
J.D.	2	4	Talipes	J.F.	27	6	Acute Respiratory Infection
T.S.	3	5	Inguinal Hernia				
R.M.	4	5	***Asthma	J.M.	28	6	Congenital Ptosis
R.M.	5	5	Tonsillitis	J.M.	29	7	Constipation
G.R.	6	6	Viral Meningitis	A.C.	30	8	Keloid
E.C.	7	6	Osteitis	C.O.	31	8	Dermatitis
W.M.	8	7	Epistaxis	F.M.	32	8	Henoch-Schonlein Purpura
A.B.	9	7	**Rheumatoid Arthritis				
J.D.	10	7	Tonsillitis	J.H.	33	8	Mycoplasma Pneumonia
B.M.	11	7	Fracture Femoral Shaft				
N.M.	12	7	Acute Pharyngitis	L.H.	34	8	Psoriasis
I.M.	13	7	Ventricular Septal Defect	I.D.	35	9	Acute Appendicitis
				C.U.	36	10	Acute Appendicitis
G.S.	14	8	Osteomyelitis	B.S.	37	10	Rickets (Nutritional-Immigrant)
M.D.	15	8	***Asthma	S.H.	38	10	**Rheumatoid Arthritis
I.W.	16	8	Erythema Multiforme	F.E.	39	10	***Acute Leukaemia
A.M.	17	8	Inguinal Hernia	L.W.	40	10	Arthralgia
C.T.	18	8	Atopic Eczema	O.V.	41	11	Talipes
K.M.	19	8	Constipation	C.R.	42	11	Acute Pharyngitis
B.M.	20	9	Tonsillitis	A.G.	43	11	Chronic Otitis Media
R.B.	21	9	Acute Pharyngitis	A.R.	44	11	Rickets (Nutritional-Immigrant)
D.S.	22	10	Cystic Fibrosis	Z.L.	45	12	Ventricular Septal Defect
				C.M.	46	12	Crohn's Disease
M.B.	23	10	***Cyclical Neutropenia	A.J.	47	12	Ventricular Septal Defect
M.C.	24	10	Traumatic Separation of Tibial Epiphysis	M.M.	48	13	Healing Multiple Leg Fractures
				D.F.	49	13	*Epilepsy
D.H.	25	11	Tricuspid Atresia with Ventricular Septal Defect	D.R.	50	14	Cystic Fibrosis

* - phenytoin ** - salicylates *** - glucocorticosteroids

children ranged in age from 3 to 11 years and the girls from 4 to 14 years. The fact that the majority of patients are in the older age groups is related to the need for 24-hour urine samples and hence ease of collection.

Patients were considered hypothyroid when the clinical status and thyroid function tests concurred. The abnormal patients investigated are listed in Table XIV. An identification number, age and biochemical status are also shown. It will be noted that more females than males were investigated and this is due to the fact that thyroid disease is allegedly more common in girls (Andersen, 1975).

The Urinary Free Thyroid Hormones in Euthyroid Children

In Tables XV and XVI are the results of the urinary free triiodothyronine and free thyroxine in the euthyroid group of patients. It will be noted that the results are recorded according to age and sex. The free urinary triiodothyronine values ranged in the male (Table XV) children from 292ng. per day (patient number 1) to 443ng. per day (patient number 25) with a mean and standard deviation of 346.4 $\pm$ 54.91ng. per day. The free urinary thyroxine in the same group ranged from 330ng. per day (patient number 11) to 597ng. per day (patient number 22) with a mean and standard deviation of 478.92 $\pm$ 81.59ng. per day. In the female children (Table XVI) the triiodothyronine values ranged from 296ng. per day (patient number 35) to 443ng. per day(patient number 43) with a mean and standard deviation of 350.68 $\pm$ 46.52ng. per day. The free

TABLE XIV : Serum total triiodothyronine (T_3), total thyroxine (T_4) and thyroid stimulating hormone (T.S.H.) in a group of hypothyroid patients

Patient Name	Patient Number	Age Years	Sex	T_3 (nmol/L)	T_4 (nmol/L)	T.S.H. (mU/L)	Clinical Diagnosis
S.B.	51	0.08	Male	1.3	37	> 100	Goitrous Hypothyroidism
A.P.	52	0.3	Male	N.A.	23	50	Primary Hypothyroidism
N.K.	53	0.5	Female	1.0	16	50	Primary Hypothyroidism
L.B.	54	1	Female	1.3	26	50	Primary Hypothyroidism
J.J.	55	2	Female	1.4	45	50	Primary Hypothyroidism
*A.M.	56	7	Female	1.1	40	< 2	Primary Hypothyroidism
S.S.	57	9	Female	0.6	15	50	Primary Hypothyroidism
*F.T.	58	11	Male	1.0	48	1.9	Secondary Hypothyroidism

* Craniopharyngioma (postoperative) was the primary pathology in all cases of secondary hypothyroidism.

N.A. - not assayed.

TABLE XV : Daily excretion of urinary free triiodothyronine (T_3) and thyroxine (T_4) and the T_4/T_3 ratio in the normal males of the series.

Name	Patient Number	Age Years	T_3 ng. per 24 hours	T_4 ng. per 24 hours	T_4/T_3
C.B.	1	3	292	446	1.5273
J.D.	2	4	337	487	1.4451
T.S.	3	5	235	352	1.4978
R.M.	4	5	284	412	1.4507
R.M.	5	5	330	466	1.4121
G.R.	6	6	311	415	1.3344
E.C.	7	6	308	350	1.1363
W.M.	8	7	358	495	1.3826
A.B.	9	7	347	426	1.2276
J.D.	10	7	372	533	1.4327
B.M.	11	7	304	330	1.0855
N.M.	12	7	393	554	1.4096
I.M.	13	7	320	574	1.7937
G.S.	14	8	270	433	1.6037
M.D.	15	8	390	417	1.0692
I.W.	16	8	333	593	1.7807
A.M.	17	8	336	562	1.6726
C.T.	18	8	415	439	1.0578
K.M.	19	8	433	558	1.2886
B.M.	20	9	369	517	1.4010
R.B.	21	9	379	579	1.5277
D.S.	22	10	429	597	1.3916
M.B.	23	10	282	442	1.5673
M.C.	24	10	396	417	1.0530
D.H.	25	11	443	579	1.3069
Mean ± S.D.			346.64 ± 54.91	478.92 ± 81.59	

TABLE XVI : Daily excretion of urinary free triiodothyronine (T_3) thyroxine (T_4) and the T_4/T_3 ratios in the normal females of the series.

Name	Patient Number	Age Years	T_3 ng. per 24 hours	T_4 ng. per 24 hours	T_4/T_3
T.M.	26	4	300	450	1.5000
J.F.	27	6	348	490	1.4080
J.M.	28	6	316	371	1.1740
J.M.	29	7	314	414	1.3180
A.C.	30	8	315	338	1.0730
C.O.	31	8	396	390	0.9848
F.M.	32	8	316	512	1.6202
J.H.	33	8	322	479	1.4875
L.H.	34	8	392	465	1.1862
I.D.	35	9	296	357	1.2060
C.U.	36	10	433	502	1.1593
B.S.	37	10	316	384	1.2151
S.H.	38	10	381	533	1.4514
F.E.	39	10	315	400	1.2698
L.W.	40	10	354	511	1.4435
O.V.	41	11	309	518	1.6763
C.R.	42	11	320	472	1.4750
A.G.	43	11	443	508	1.1467
A.R.	44	11	313	575	1.8370
Z.L.	45	12	401	676	1.6857
C.M.	46	12	315	475	1.5079
A.J.	47	12	340	555	1.6323
M.M.	48	13	413	662	1.6029
D.F.	49	13	373	560	1.5013
D.R.	50	14	426	620	1.4553
Mean ± S.D.			250.68 ± 46.5	488.68 ± 89.99	

urinary thyroxine in this group ranged from 338ng. per day (patient number 30) to 620ng. per day (patient number 50) with a mean and standard deviation of 488.68 ± 89.99ng. per day. There is no statistically significant difference between any of these values. The mean values are summarised in Table XVII for the sexes and for the whole group.

TABLE XVII : Urinary triiodothyronine (T_3) and thyroxine (T_4) levels estimated by gas liquid chromatography in euthyroid children. Mean ± S.D.

Subjects	T_3 ng per 24-hours	T_4 ng per 24-hours
Male euthyroid children	346.64 ± 54.91	478.92 ± 81.59
Female euthyroid children	350.68 ± 46.52	488.68 ± 89.99
All children	348.66 ± 50.41	484.20 ± 85.15

Patients number 49; 9 and 38 and 4, 15, 23, 29 were receiving phenytoin, salicylates and corticosteroids respectively. I note this here for there is some evidence that those drugs may alter the binding capacity of thyroxine-binding-globulin or its concentration. However the values for urinary triiodothyronine and thyroxine in these patients are within the normal range.

From the above data a number of main conclusions may be drawn. Firstly for the whole group investigated the urinary triiodothyronine and thyroxine values cluster closely, indeed more closely than those values reported in the literature for adults. Secondly the thyroxine

excretion rate is greater on average than that for triiodothyronine. Thirdly the measured urinary thyroid hormones did not seem to be affected by the drugs which are known to alter the protein-binding concentration or capacity since in the seven cases on such drugs, gross deviations were not detected. On the other hand I was not able to determine the thyroxine-binding globulin and thyroxine-binding peralbumin in these patients. Nonetheless the findings are in agreement with those of other workers (Chan, 1974; Pastrana et al., 1975; Burke & Shakespear, 1975) who could not detect significant differences in urinary triiodothyronine and thyroxine in adults on treatment with these drugs.

My mean values for the daily urinary excretion rate for free triiodothyronine and free thyroxine in euthyroid children are less than all those previously reported for adults. Those who have published have however only studied adults and measured rather than total (bound + free) urinary thyroid hormones (Burke & Shakespear, 1976) and not the free moitie. In Tables XVIII and XIX are those published adult values which were determined either by radioimmunoassay or competitive binding techniques. I have chosen to compare my results with those of Black et al.,(1975) because of all the authors they alone have estimated by radioimmunoassay both triiodothyronine and thyroxine in the urine (without extraction) in a sizeable number (24) of adult patients (Table XX).

TABLE XVIII : Urinary levels of triiodothyronine in euthyroid adults (A review of the literature)

Author(s)	Triiodothyronine (ug. per 24 hours)		Method of Quantitation
	Mean ± S.D.	Range	
Chan et al., 1972	2.9 ± 0.5	2.0 - 4.5	RIA after ethyl acetate extraction
Burke et al., 1972	0.8	0.33 - 1.91	Direct RIA
Hefner & Hesch, 1973		0.4 - 2.4	Direct RIA
Chan et al., 1973	2.9 ± 0.5		RIA after ethyl acetate extraction
Burke et al., 1973	0.8	0.33 - 1.91	Direct RIA
Chan, 1974		0.8 - 2.4	RIA after ethyl acetate extraction
Rastogi et al., 1974	7.0*		Direct RIA
Burke & Shakespear, 1975	1.1*		Direct RIA
Gaitan et al., 1975	1.0 ± 0.34	0.5 - 1.83	Direct RIA
Black et al., 1975	0.95±0.33	0.5 - 1.73	Direct RIA
Shakespear & Burke, 1976	0.82**		Direct RIA
Finucane et al., 1977	1.07 ±0.34	0.66- 1.73	Direct RIA

* calculated from their graph.

** original values were given in ng. per hour.

RIA = Radioimmunoassay.

TABLE XIX : Urinary levels of thyroxine in euthyroid adults

(a review of the literature)

Author(s)	Thyroxine (ug. per 24 hours)		Method of Quantitation
	Mean ± S.D.	Range	
Chan & Landon, 1972	8.3 ± 2.2	4.3 - 12.7	CPB protein binding after ethyl acetate extraction
Burke et al., 1972	2.0	0.54 - 3.9	CPB on "Sephadex" column
Chan et al., 1972	8.0 ± 2.1		CPB after ethyl acetate extraction
Burke et al., 1973	2.0	0.54 - 3.9	CPB on "Sephadex" column
Chan et al., 1973	8.0 ± 2.1		CPB after ethyl acetate extraction
Chan, 1974		4.3 - 12.7	CPB after ethyl acetate extraction
Rastogi et al., 1974	15.0*		Direct RIA
Pastrana et al., 1974	2.1	0.6 - 3.6	Direct RIA
Pastrana et al., 1975	2.0	2.5 - 3.5	Direct RIA
Black et al., 1975	2.0 ± 0.63		CPB on "Sephadex" column
Black et al., 1975	1.21± 0.49	0.44 - 2.05	Direct RIA
Shakespear & Burke, 1976	1.9**		CPB on "Sephadex" column
Shakespear & Burke, 1976	2.0**		Direct RIA
Finucane et al., 1977	1.95± 0.62	0.85 - 3.08	Direct RIA

* Calculated from their graph

** Original values were given in ng. per hour

RIA - Radioimmunoassay.

CPB - Competitive protein binding.

TABLE XX : Urinary levels of triiodothyronine and thyroxine in euthyroid children and adults. A comparison based on different methodologies.

Compound	Method of Assay and Author(s)		
	GLC (children) own results	RIA (adults) Black et al., 1975	CPB (adults) Black et al., 1975
Triiodothyronine (ug. per 24 hours)	0.34 ± 0.05 (n = 50)	0.95 ± 0.33 (n = 24)	
Thyroxine (ug. per 24 hours)	0.48 ± 0.08 (n = 50)	1.21 ± 0.49 (n = 24)	2.0 ± 0.63 (n = 23)

GLC - Gas liquid chromatography

RIA - Radioimmunoassay

CPB - Competitive protein binding

My values are approximately one-third of those of Black et al., (1975). This is not to be considered serious since there is considerable controversy regarding the specificity of the competitive protein binding and radioimmunoassay methods when applied to extracted or even unextracted urine. Indeed the competitive protein binding methods give grosser results for urinary thyroxine than the radioimmunoassay method and it has been condemned by Black et al., (1973) largely because a number of thyroxine peptide derivatives displace thyroxine from binding globulin (Tabachnick et al., 1971). The competitive protein binding method overestimates by 60 per cent the values of thyroxine found by the radioimmunoassay method (Black et al., 1975). Using the radioimmunoassay methods for triiodothyronine and thyroxine in unextracted urine, the disparity between the values

reported by different groups of workers (Tables XVIII and XIX) indicates that the problem of cross-reaction with the hormone metabolites in urine is not yet solved. Additionally the radioimmunoassay methods estimate protein bound and free hormone without discrimination. Using steady-state gel filtration of normal adult urine, Burke & Shakespear (1976) estimated that only 40 per cent and 20 per cent of triiodothyronine and thyroxine respectively estimated either by the radioimmunoassay or competitive protein binding methods are in the free form. The calculated ~~estimated~~ values of the latter authors for the free urinary thyroid hormones are similar to those reported in this work as determined by the developed methodology (Table XXI).

TABLE XXI : Free urinary triiodothyronine and thyroxine levels in euthyroid children and adults. (Levels of free hormones in adults calculated from author's data.

Compound	Method of Assay		
	GLC(children) own results	RIA (adults) Shakespear & Burke, 1976	Calculated* Free (adults) Burke & Shakespear, 1976
Triiodothyronine (ng. per hour)	14.52 ± 2.09 (n = 50)	34.3 ± 15.4 (n = 38)	13.72 (40% of total)
Thyroxine (ng. per hour)	20.17 ± 3.60 (n = 50)	83.2 ± 31.4 (n = 6)	16.74 (20% of total)

* Calculated from Column III on the finding of Burke & Shakespear, 1976 that only 40% of the estimated triiodothyronine and 20% of thyroxine by radioimmunoassay are in the free form.

The gas liquid chromatography methodology, while prolix, has no cross-reacting artefacts and is highly specific and reproducible. The thyroid hormones were identified by their retention times

Compared with those of purified standards. Although it is theoretically possible that two compounds may share the same retention time under certain conditions of column and temperature, this is less likely in the temperature programmed technique adopted in this work. Thus although tedious the gas liquid chromatography methodology appears to offer more specificity and less interference than either the competitive protein binding or the radioimmunoassay methods.

Upward Trends in Urinary Thyroid Hormones with Age

It is of interest that when the patients are grouped according to age (Table XXII) and the mean urinary values of triiodothyronine and thyroxine calculated, there appears to be an upward trend with age, although statistically the differences between the groups are not significant. Since it was earlier pointed out that there was no gross difference in the urinary free triiodothyronine and thyroxine between the youngest and oldest patient in the cohort, significant differences in the means of age groups was not expected. If it is true that there is a small increase in the free hormone excretion with age, it may be that this represents a changing renal function with age and this possibility will be debated later.

TABLE XXII : Changes in the daily excretion of urinary thyroid hormones associated with age (ng. per 24 hours).

Mean ± S.D. (and range) for the whole series.

Age Group	Triiodothyronine		Thyroxine	
	Mean ± S.D.	(Range)	Mean ± S.D.	(Range)
3 - 5 years (n = 6)	296.33±36.69	(235-337)	435.5±47.80	(352-487)
6 - 10 years (n = 33)	350.27±45.14	(270-433)	465.96±83.65	(330-597)
11 - 14 years (n = 11)	371.45±61.117	(309-443)	511.0±258.76	(472-620)

Ratio of Urinary Free Thyroxine to Free Triiodothyronine

The ratio of urinary free thyroxine to free triiodothyronine was calculated for each child (Tables XV and XVI). The same age groups as in Table XXII are taken and means and standard deviations calculated (Table XXIII). The overall mean ratio is 1.348 to 1 with a range from 1.05 to 2.41.

TABLE XXIII : Ratio of urinary free thyroxine to free triiodothyronine in euthyroid children according to age group.

Age Group (Years)	Number of Patients	Mean	Range
3-5	6	1.4721	1.4121 - 1.5273
6-10	33	1.2649	0.9848 - 1.7937
11-14	11	1.5297	1.1467 - 1.8370

However there may be some significance in the fact that in the children less than 6 years the mean ratio of free thyroxine to triiodothyronine (mean 1.4721) is greater than that for the age range 6 to 10 years (mean 1.2649). Again in the immediately prepubertal and puberty age range the ratio of urinary free thyroxine to triiodothyronine rises again (mean 1.5297). However as with the actual excretion values for thyroxine and triiodothyronine there is here also an overlap particularly of the middle group with the means for the younger and older age groups. Clearly, a changing ratio, albeit within narrow limits, could be due to changes in the excretion rates of either thyroxine or triiodothyronine and since I have shown an upward trend in the excretion rates for both free thyroxine and triiodothyronine, any changes in the excretion ratios must be related to the mechanisms of renal handling of both substances. There is a paucity of data in the literature capable of being brought together for comparison with my own results but I will bring together what seems valid to assist with the interpreting of my results presented here.

Let me first speculate on my suggestion that there is an upward trend in the excretion of urinary free thyroxine and triiodothyronine with age. According to several authors the daily urinary output of triiodothyronine and thyroxine in the neonate is less than that found in the adult (Table XXIV), and it therefore seems likely that a smooth increase throughout the paediatric age would probably be definable were samples from more patients between these extremes of age available for study.

TABLE XXIV : Comparison of daily excretion of thyroid hormones (ug. per 24 hours) in neonates with adults. A review of the literature.

AUTHORS	TRIIODOTHYRONINE		THYROXINE	
	Neonates	Adults	Neonates	Adults
Chan et al., 1972				
No. of patients	5	44		
Mean ± S.D.		2.9±0.5		
Range	(0.1-0.4)			
Chan et al., 1973				
No. of patients	20	24	20	24
Mean ± S.D.	0.18±0.08	2.9±0.5	0.83±5.6	8.0±2.1
Range	(0.02-0.36)		(0.16-2.1)	
Burke et al., 1973				
No. of patients				
Mean ± S.D.			0.86	2.0
Range				(0.54-3.9)

In general, the level of urinary free thyroid hormones represents the end product of an equation which includes secretion, the prevailing serum unbound level, protein-binding, target organ utilisation, liver metabolism and renal handling. It is not yet clear what role the kidney plays comprehensively in this system. However, we know that the glomerular filtration rate adjusted for body surface area is low in infancy, reaching the adult normal value of 120 ± 20ml. per minute per 1.73 M^2 (the body surface area of a "standard adult") in early childhood (Rahill, 1969). Again, at birth the corrected clearance of inulin (indirect measurement of

glomerular filtration rate) varies between 10 and 50 per cent of the normal for older (6-18 month) children. Then a progressive rise in filtration rate occurs from birth to the age of 6 to 18 months, when the childhood norm is attained. Additionally the para-aminohippurate clearance (indirect estimation of effective renal plasma flow) at low plasma levels of para-aminohippurate (5mg per 100ml.) is low in relation to body surface in infancy and only reaches the adult value of 600ml. per minute per 1.73 M^2 at about 2 years of age. One therefore concludes that there is a relatively low effective renal perfusion during infancy. Thus both the low glomerular filtration rate and the low renal perfusion rate of early infancy could result in the reduced output of urinary thyroid hormones during the first week of life and this despite their high free serum levels.

The mean serum free thyroxine concentration shortly after birth (1-3 days) is 8.6ng. per 100ml. (Marks et al., 1966). Subsequently it decreases during the first week of life and remains constant at a mean concentration of 4.7ng. per 100ml. (Lemarchand-Beraud et al., 1972) until approximately 12 years of age. For what the values are worth the basal metabolic rate declines steadily from birth to adulthood, except at puberty when there is a temporary rise. If the falling basal metabolic rate (BMR) indicates a reduced need for free serum thyroid hormones and if the gland output remains steady, then to maintain a constant level of free thyroxine one might expect an increase in renal loss. The situation is similar with regard to the total serum thyroxine. The total serum thyroxine as opposed to the free thyroxine is well recognised as falling

slightly but significantly during childhood and thus once again if production remains constant then the excretion must increase. From another point the serum protein bound iodine falls during childhood (Fisher, 1971) yet if the serum free thyroxine level remains constant then this fall must be due to a reduction of the thyroid hormone binding proteins. This too is a known fact.

Thus a smooth increase in the urinary excretion of thyroxine could explain the steady state of the free serum thyroxine level during childhood although only if the thyroxine production remains constant. Until this moment no published values for the free serum triiodothyronine levels in childhood are reported.

Now at puberty there is a slight increase in the basal metabolic rate (Tanner, 1962). This might lead one to suppose that the thyroid gland becomes more active during this period. Serum thyrotrophin levels measured by radioimmunoassay, declining gradually throughout childhood, are at adolescence (i.e. 18 years) still above young adult levels. There is probably a temporary increase in thyrotrophin levels at puberty, especially in girls, though the available data are as yet insufficiently precise to establish this with certainty (Goldstein-Golaire & Delange, 1971; Lamburg et al., 1973b). Certainly the thyroid uptake of radio-iodine is increased during puberty (Malvaux et al., 1965; Delange & Ermans, 1967). The implication therefore is that thyroid function does increase somewhat at puberty. Does this explain the common occurrence of pubertal goitre in girls?

To account for the rise in both the excretion rates and thyroxine to triiodothyronine ratio at puberty, let us consider the

changes in binding proteins at this age. There is a suggestion from current data (Rauth et al., 1964; Oddie & Fisher, 1967; Goldsmith et al., 1967; Stekelenburg et al., 1970; Lamburg et al., 1973a) that there is a slight fall in both the serum protein bound iodine and the total thyroxine coincident with the adolescent growth spurt. After puberty there is a recovery to the childhood levels. It is thought that this may not represent an adolescent decrease in the secretion rate of thyroxine, but rather a decrease in the amount of proteins which carries the thyroxine in the blood. These carriers are thyroxine-binding globulin (TBG) and thyroxine-binding peralbumin (TBPA). TBG declines steadily during childhood and reaches adult levels, at least in girls by the end of adolescence (18 years). From then on there is no change (Lamburg et al., 1973a). In boys, however, Goldsmith et al. (1967) found an abrupt temporary decrease in thyroxine-binding globulin at puberty, perhaps owing to androgen action for it is known that with androgen therapy there is an induced fall in thyroxine-binding globulin levels. Thyroxine-binding peralbumin concentration rises in late puberty (Goldsmith et al., 1967). No significant changes of total serum triiodothyronine occur during puberty (Lamburg et al., 1973a; Ruskin et al., 1973). Thus if there is a fall in plasma binding proteins within the pubertal period then it is easy to appreciate that there may also be an increased urinary loss of free thyroxine since both the thyroid production and the serum levels of the free thyroxine remain constant. Thus the changes in the urinary thyroxine to triiodothyronine ratio are likely to be due to changes in the free thyroxine excretion at the different ages.

As stated earlier, there are no significant changes in serum free thyroxine levels and one therefore assumes that triiodothyronine both in the circulation and urine is the constant.

My conclusion from my own results is that there is a slight but measurable upward trend in urinary free triiodothyronine and thyroxine through the paediatric age group.

We come now to consider the renal handling of the available free plasma hormones in relation to the changing needs of a growing child. Unfortunately, the precise mechanism of renal handling of thyroid hormones is not yet known nor are the free serum levels of thyroxine and triiodothyronine yet agreed. This is due to the non-specificity of the methods used. Additionally there are no reported values for free serum triiodothyronine in the paediatric age range. Adult values for both free serum thyroxine and triiodothyronine are available. The adult free thyroxine values reported are less than those in the paediatric age (Hung, 1975). For the purpose of comparison it is reasonable to assume that the paediatric serum free triiodothyronine level will also be higher than the adult value although the thyroxine to triiodothyronine ratio in both adult and child is likely to be similar. This assumption will be used for assessment of the urinary free thyroxine to triiodothyronine ratios which I have found here.

In euthyroid subjects the mean ratio of urinary free thyroxine to free triiodothyronine as determined in this work by gas liquid chromatography is 1.348 to 1. Lower values of 1.27 (Black et al., 1975) and higher values of 1.82 (Finucane et al., 1977) have been reported in adults. Table XXV gives the adult urinary thyroxine

to triiodothyronine ratios calculated from values of urinary free thyroxine and triiodothyronine found in the literature.

TABLE XXV : Mean urinary thyroxine (T_4) to triiodothyronine (T_3) ratio in euthyroid adults. (Review of the literature).

AUTHOR(S)	MEAN T_4 (ug. per day)	MEAN T_3 (ug. per day)	MEAN T_4 / MEAN T_3 ratio
Chan et al., 1972	8.0	2.9	2.75
Burke et al., 1972	2.0	0.8	2.5
Black et al., 1975	1.21	0.95	1.27
Shakespear & Burke, 1976	82.2*	34.3*	2.39
Finucane et al., 1977	1.95	1.07	1.82

* ng. per hour.

The ratio of serum free thyroxine to free triiodothyronine is 5.4 (Ellis & Ekins, 1973). Let us assume that this ratio also obtains in children. Comparing this ratio with the urinary free thyroxine to free triiodothyronine ratio which I have established, there has been a change from serum to urine of 5/1 to 1/1 for thyroxine to triiodothyronine.

Let us consider in detail the renal handling of both hormones.

Thyroxine

If we take the mean value of free serum thyroxine in childhood as 4.7ng. per 100ml. (Lemarchand-Beraud et al., 1972) a mean

filtered load of thyroxine of 5.64ng. per minute ($\frac{4.7 \times 120}{100}$ ng. per minute) (assuming a glomerular filtration rate of 120ml. per minute) is presented to the tubules. If we now take my overall mean urinary excretion rate for thyroxine of 0.3362 ng. per minute (484.2 ng. per 24-hours) then the percentage tubular reabsorption can be calculated as under:-

Mean free serum thyroxine = 4.7ng. per 100ml.

Mean glomerular filtration rate = 120ml. per minute

Mean filtered load of thyroxine = 5.64ng. per minute

Mean excreted thyroxine per minute = 0.3362ng. per minute

Mean tubular reabsorption (per cent) =

$$\frac{\text{Filtered thyroxine} - \text{Excreted thyroxine}}{\text{Filtered thyroxine}} \times 100$$

$$\frac{5.64 - 0.3362}{5.64} \times 100 = 94.03 \text{ per cent}$$

This figure is consistent with the behaviour of the low molecular weight solutes which are almost entirely reabsorbed. For example free cortisol has a reabsorption rate by the tubules of 98 per cent (Beisel et al., 1964).

Burke & Shakespear (1976) gives a tubular reabsorption rate of 65 per cent for thyroxine but this is based on their estimates of the total urinary thyroxine (bound and free). However, using steady-state gel filtration they show that only 20 per cent of the total urinary thyroxine is free, the remainder being protein-bound (12 per cent) or bound to low molecular weight binder (68 per cent). The authors further suggest that the protein-bound thyroxine in the urine is leaked out protein-bound thyroxine through the glomerulus.

Had they similarly calculated the tubular reabsorption of thyroxine on their value for the free urinary thyroxine (mean serum unbound thyroxine 39.6ng. per litre, mean total urinary excretion 82.2ng. per hour and glomerular filtration rate 120ml.) then their tubular reabsorption index for thyroxine would have been 94.23 per cent which agrees with my own figure. That these final figures agree lends credence to our separate methodologies.

Triiodothyronine

Again there is no value for free serum triiodothyronine for paediatric patients but Burke & Shakespear (1976) have given adult values (mean 4.65ng. per litre). If we take these as probably not too disparate from those of children, then I can calculate the tubular reabsorption rate of triiodothyronine for my patients. The mean excretion rate of triiodothyronine in my series is 348.66ng. per 24-hours or 0.2421ng. per minute. A free serum triiodothyronine level of 4.65ng. per litre is equivalent to a filtered load of 0.558ng. per minute assuming a mean glomerular filtration rate of 120ml. per minute.

Thus the tubular reabsorption of triiodothyronine:

$$= \frac{\text{Filtered load per minute} - \text{Excretion rate per minute}}{\text{Filtered load per minute}} \times 100$$

$$= \frac{0.558 - 0.2421}{0.558} \times 100 \qquad = 56.61 \text{ per cent.}$$

Burke & Shakespear (1976) do not calculate the tubular reabsorption of triiodothyronine because they found that their mean

urinary excretion rate (0.570ng. per minute) was greater than the calculated triiodothyronine load presented to the tubules by a factor of 1.26. Thus not only is all the filtered triiodothyronine lost but there must be an active secretion of triiodothyronine in addition. One naturally doubts if this is so. In fact, using steady-state gel filtration they show that only 40 per cent of their total urinary triiodothyronine is free, the remainder being protein-bound (9 per cent) or bound to low molecular weight binder (50 per cent). Had they calculated the tubular reabsorption of triiodothyronine on the free urinary triiodothyronine (0.2286ng. per minute, i.e. 40 per cent of 0.570) then their tubular reabsorption index would have been 59.03 per cent. This figure agrees with my own finding and once again supports the accuracy of my methodology.

If triiodothyronine is reabsorbed from the renal tubules according to my value of 56.61 per cent (tubular reabsorption rate) then there is a gross difference between the tubular reabsorption of thyroxine (94.03 per cent) or cortisol (98 per cent) and of triiodothyronine (56.61 per cent). One would have expected that triiodothyronine being also a low molecular weight solute would have behaved like thyroxine and cortisol.

This phenomena is partially explained by a major metabolic event affecting thyroxine at the renal level. The capacity of isolated human kidney tissue to convert thyroxine to triiodothyronine has long been recognised (Albright et al., 1954) and the physiological importance of thyroxine monodeiodination is well established (Braverman et al., 1970). From the figures provided by Pittman et al.,

(1971) it appears that about one-third of the total thyroxine production is transformed to triiodothyronine by peripheral monodeiodination which accounts for 40 per cent of the total triiodothyronine pool (Hoffenberg, 1973). However the rate of thyroxine monodeiodination at the renal level is not yet known. But if this thyroxine monodeiodination does also occur to the filtered thyroxine at the tubular wall (Gaitan et al., 1975; Burke et al., 1972; Finucane, 1976) and the formed triiodothyronine is excreted, then the measured triiodothyronine consists of a normally filtered moitie plus that derived from thyroxine in transit.

On this basis let us now consider the possible significance of renal transformation of thyroxine to triiodothyronine on the tubular reabsorption rates for triiodothyronine and thyroxine.

If 30 per cent of thyroxine is transformed to triiodothyronine during ultrafiltration, then 30 per cent of the filtered thyroxine will appear in the tubule as triiodothyronine.

Thus with a glomerular filtered load of thyroxine of 5.64 ng. per minute there is a conversion to triiodothyronine of $\frac{5.64}{3}$ ng. per minute i.e. 1.87 ng. per minute.

Thus the filtered triiodothyronine (0.558 ng. per minute) in the tubule is now augmented by 1.87ng. per minute i.e. to 2.428ng. per minute.

Using this figure to calculate the tubular reabsorption of triiodothyronine, the calculated value is:

$$\frac{2.428 - 0.2421}{2.428} \times 100 = 90.02 \text{ per cent}$$

This is similar to that calculated for thyroxine and is more in agreement with reabsorption rate for low molecular weight solutes. A small reduction in this figure would be obtained if

the relative weights of triiodothyronine and thyroxine were taken into account. Thus using the published plasma levels for the free hormones in conjunction with my own values of free urinary triiodothyronine and thyroxine, I find that the tubular reabsorption for both triiodothyronine and thyroxine are similar and both above 90 per cent. This finding is in keeping with the behaviour of low molecular weight ultrafiltrable solutes.

Renal Handling of Thyroid Hormones with Age

It is not yet clear what is the contribution of renal metabolism of these hormones to the values estimated. The kidney plays an important role in the peripheral metabolism of iodine and thyroid hormones and the thyroid hormones play an important role in kidney growth and function (Katz et al., 1975). However, there may be some significance in the fact that in the 0 - 5 year age range, the mean ratio of thyroxine to triiodothyronine is greater than 1.4 (1.47) while for the age range 6 - 10 the ratio is less than 1.4 (1.26). Again in the immediately prepubertal and puberty age the ratio of urinary free thyroxine to triiodothyronine rises again to be greater than 1.4 (1.52). Thus it seems that less free triiodothyronine is excreted when the metabolic activity is likely to be increased because of the rapid changes in linear growth (in the younger) and at puberty in the older, while the intermediate age group is relatively coasting. It is true that the renal excretion of triiodothyronine and thyroxine represent the end result of a metabolic equation, however the variables of this equation are

changing according to the needs of a growing body.

The accepted constant mean level of free serum thyroxine during childhood (Marks et al., 1966; Lemarchand-Beraud et al., 1972), with the declining protein-bound iodine throughout this period (Fisher, 1971), the increasing thyroid weight with age (Kay et al., 1966) and the normal growth velocity and basal metabolic rate strongly suggest a major role for the monodeiodination rate of thyroxine to triiodothyronine. This rate is probably not constant throughout the growing period but is adapted to supply the more active hormone viz. triiodothyronine according to the body needs. This conclusion is supported by the increasing evidence of the great part played by triiodothyronine in thyroid physiology.

Considering the total thyroid hormone metabolic activity there is a greater contribution from triiodothyronine on the basis that triiodothyronine is approximately three times more active than thyroxine. Thus of the daily turnover of triiodothyronine and thyroxine of 60ug. and 80ug. respectively, approximately two-thirds of the total metabolic effect comes from triiodothyronine (i.e. 60 x 3 of 80 or $\frac{180}{260}$ i.e. $^2/_3$). In addition the clinical usefulness of the triiodothyronine suppression test presuppcses a direct action of triiodothyronine in achieving inhibition of thyrotrophin response to thyrotrophin releasing hormone (Bowers et al., 1971). Thyroxine alone can achieve the same effect (Greer & Smith, 1954) but one must now ask whether this is a direct action or one exerted through its preliminary transformation to triiodothyronine. It is interesting to mention that human pituitary tissues have been shown to deiodinate thyroxine to produce triiodothyronone (Volpert

et al., 1963).

Thus it might be assumed that changes in the rate of monodeiodination of thyroxine to triiodothyronine as well as changes in the tubular reabsorption of both hormones combine to meet the changing needs of the growing body as it passes from a phase of rapid growth in the first four years through a period of steady growth then into the pubertal growth spurt. These changes in tubular reabsorption I think I have detected in the changing excretion rates of free urinary thyroxine and triiodothyronine. Obviously this conclusion could be confirmed if the plasma levels of both free triiodothyronine and thyroxine were available for each age group.

Urinary Free Thyroid Hormones in Hypothyroid Patients

In this work a number of patients with hypothyroidism were available for study. Table XXVI shows their urinary free triiodothyronine and thyroxine values as determined by gas liquid chromatography. Triiodothyronine values ranged from undetectable to 178ng. per 24 hours and thyroxine values from undetectable to 247ng. per 24 hours. It will be noted that in two patients triiodothyronine could not be detected and in five, thyroxine could not be detected. If the reader refers again to Table XIV (page 99) he will observe that even although the patients were clinically and biochemically subthyroid, a low level of serum thyroid hormones were detected by radioimmunoassay, only in those patients whose serum thyroxine was in excess of 40 nmol. per litre

did thyroxine leak out in the urine sufficiently to be measured. Alternatively with serum levels less than 40 nmol. per litre there may be complete conversion of the thyroxine to triiodothyronine by the kidney so that by tubular reabsorption of the latter, at least triiodothyronine would be conserved against the failing metabolic state. It is of note that in all but two patients in Table XIV the serum triiodothyronine levels were within the normal range. Thus triiodothyronine, like thyroxine, probably only leaks out in the urine when the serum level is above a certain minimum. I have not enough data on which to adduce the significant minimum serum triiodothyronine level when urinary excretion may occur. Sterling & Lazarus (1977) in their statement that in hypothyroidism there is a preferential production of triiodothyronine, supportSmy conclusion that the kidney may play an important role in the conservancy of triiodothyronine in hypothyroid states.

TABLE XXVI : Daily excretion of urinary triiodothyronine and thyroxine (ng. per 24 hours) in hypothyroid children by age and sex.

Patient	Patient Number	Age Years	Sex	Triiodothyronine	Thyroxine
S.B.	51	0.08	Male	-	-
A.P.	52	0.3	Male	110	-
N.K.	53	0.5	Female	132	-
L.B.	54	1	Female	155	-
J.J.	55	2	Female	160	231
A.M.	56	7	Female	120	163
S.S.	57	9	Female	-	-
F.T.	58	11	Male	178	247

When the thyroid hormone is not detected it is assumed that it is either absent or below the limit of the assay. Nonetheless the highest urinary level detected in these hypothyroid patients was lower than the lowest value in the normal subjects, thus there was no overlap noted between the excretion values for the normal and the subthyroid patients. Others (Black et al., 1975; Gaitan et al., 1975; Shakespear & Burke, 1976) (Table XVII) who have studied this problem in adults have found an overlap in the excretion values but these authors have used either radioimmunoassay or competitive protein-binding techniques. I have already argued against the reliability of these methods of assay (page 106).

TABLE XVII : Urinary levels of thyroid hormones in hypothyroid adults. (Review of the literature).

TRIIODOTHYRONINE (ng. per 24 hours)

Author(s)	Mean ± S.D.	Range
Chan et al., 1972	0.9 ± 0.5	0.3 - 1.6
Burke et al., 1973	0.15	
Hufner & Hesch, 1973	0.3	
Chan, 1974		0.02 - 0.7
Gaitan et al., 1975	0.36 ± 0.2	0.11 - 0.78
Black et al., 1975		0.18 - 0.53
Shakespear & Burke, 1976	0.43 *	
	0.88 **	

* Primary hypothyroid patients; original values were given in ng. per hour.

** Secondary hypothyroid patients; original values were given in ng. per hour.

Cont/ TABLE XVII :

THYROXINE (ug. per 24 hours)

Author(s)	Mean ± S.D.	Range
Chan & Landon, 1972	2.80 ± 1.7	4.2
Chan et al., 1972	2.80 ± 0.9	
Pastrana et al., 1974	0.60	0.25 - 1.5
Pastrana et al., 1975	0.65	0.1 - 1.5
Black et al., 1975		0.2 - 0.78
Shakespear & Burke, 1976	0.78 *	
	1.29 **	
Shakespear & Burke, 1976	0.41	

* Primary hypothyroid patients; original values were given in ng. per hour.

** Secondary hypothyroid patients; original values were given in ng. per hour.

The actual time taken to analyse a urine sample has been estimated at 5 days continuously working but within that period four other urine samples can be taken nearly to completion. The methodology would therefore be ideal for team-work where several technicians work on a "conveyor-belt" system and since radioactive tracers are incorporated, good checks can be maintained by the supervisor. On the other hand Black et al., (1975) claims that up to 70 urines in duplicate can be processed by radioimmunoassay within a working day. If inprecision of assay is accepted as a penalty for speed, then I have sacrificed speed in the hope that I have accuracy and specificity.

Two children with primary hypothyroidism are of interest.

Their case histories follow:

S.B. (patient 51); this is the sixth child of healthy Pakistani parents who are first cousins. Their first child and the patient both have talipes equinovarus. The pregnancy was normal and no drug had been taken by the mother. The perinatal period was normal and the birth weight was 3.5kg. The bilateral talipes noted at birth caused the infant to be transferred to the Orthopaedic Ward. While there the infant became lethargic, difficult to feed and developed a coarse cry. A small soft homogenous symmetrical goitre was noted in association with an umbilical hernia. Although the child was not clinically abnormally subthyroid the biochemical finding indicated primary thyroid insufficiency. There was epiphyseal dysgenesis. The biochemical data were as under:

	Patient	Normal Range
Total serum thyroxine	41 nmol. per litre	(55-144)
Total serum triiodothyronine	1.4 nmol. per litre	(0.9-2.8)
Thyroid stimulating hormone	50 mU per litre	(up to 8.8)

No treatment was started until the infant came to the medical wards and at age one month the following biochemical data were obtained:

	Patient	Normal Range
Total serum thyroxine	37 nmol. per litre	(55-144)
Total serum triiodothyronine	1.3 nmol. per litre	(0.9-2.8)
Thyroid stimulating hormone	100 mU per litre	(up to 8.8)

At this stage urine was collected for analyses (Figure 28).

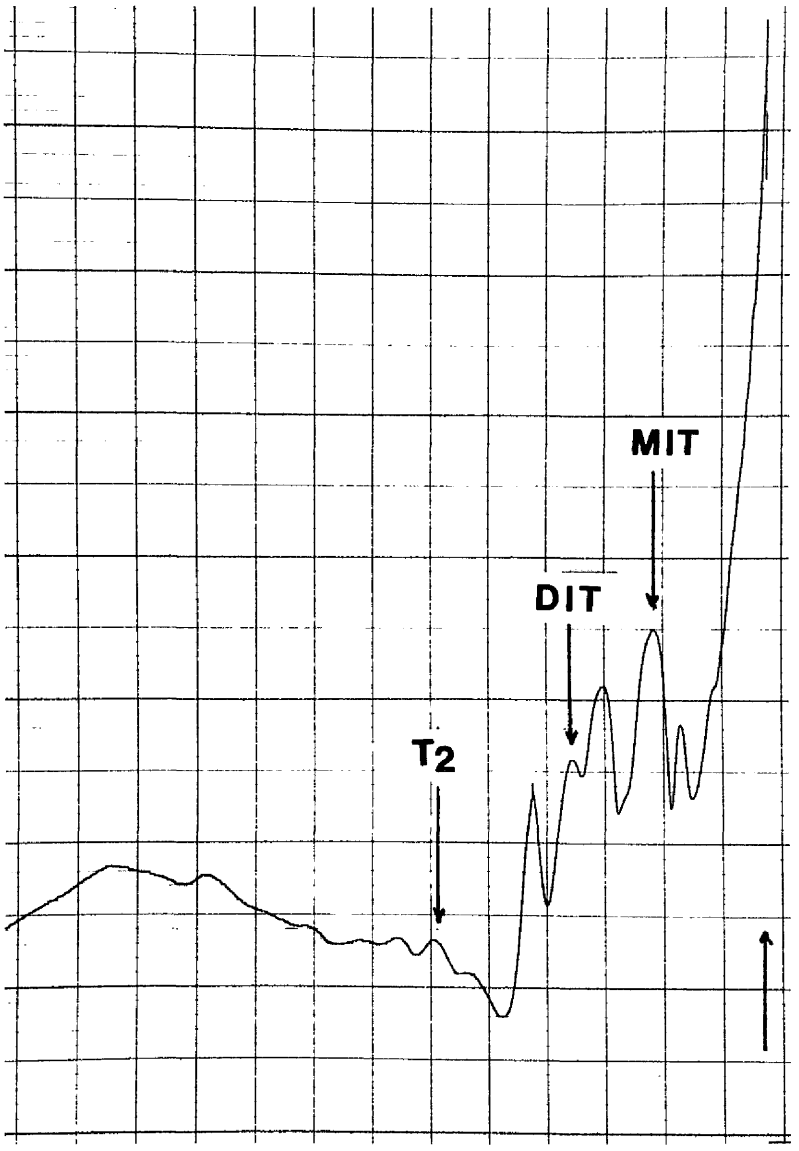


Figure 28 : Tracing of urine extract from patient S.B. (patient 51).

S.S. (patient 57); this is the middle of three children born to healthy Egyptian parents who are first cousins. The pregnancy was normal and delivery was in Gamera Hospital, Cairo. The neonatal period was normal and the milestones were reached at average time. About age 15 months the child seemed to slow down in her development and in Cairo thyroxine sodium 0.1mg. daily was given continuously until age 9 years. When seen at 9 years the complaint was of smallness of stature, lethargy and a poor school performance. Clinically she was not obviously subthyroid nor was there any thyroid swelling. The bone age was that of a 5.6 year old girl. The pituitary fossa was large. There was no epiphyseal dysgenesis. Thyroxine was discontinued for one month and thereafter the following biochemical data were obtained:

	Patient	Normal Range
Total serum thyroxine	15 nmol. per litre	(55-144)
Total serum triiodothyronine	0.6 nmol. per litre	(0.9-2.8)
Thyroid stimulating hormone	50 mU per litre	(up to 8.8)

Additionally the BEI^{131} was 29 per cent of protein bound iodine. Also the gland uptake was as follows:

1 hour	2.8 per cent
2 hours	17.1 per cent
4 hours	45.2 per cent
24 hours	69.? per cent
48 hours	58.1 per cent

At 48 hours the total plasma ^{131}I was 0.674 per cent of the total dose per litre and PBI^{131} was 0.521 per cent of the dose per litre. The thyroid scan was normal and there were no autoimmune

antibodies. A urine collection was made at this stage for study (Figure 29).

Significantly in the tracings of these two patients neither triiodothyronine nor thyroxine is seen. However monoiodotyrosine and diiodotyrosine are present in reasonable amounts. While my method was not designed to study the behaviour or quantitation of monoiodotyrosine and diiodotyrosine, I can only make a reasonable conclusion against these findings. In the light of the clinical data, it could be assumed that the excess of monoiodotyrosine and diiodotyrosine and absence of triiodothyronine and thyroxine may explain a state of failure of utilisation of monoiodotyrosine and diiodotyrosine in normal biosynthesis and hence loss in urine. This disordered metabolism is consistent with a dehalogenase defect. Clearly the solution of this type of problem will easily be found with a modification of the method (as pointed out earlier in Discussion of Methodology, page 95) so as to allow accurate quantitation of urinary thyroid hormone metabolites. A fuller understanding of thyroid hormone metabolism may well derive from further experience with this gas liquid chromatography methodology.

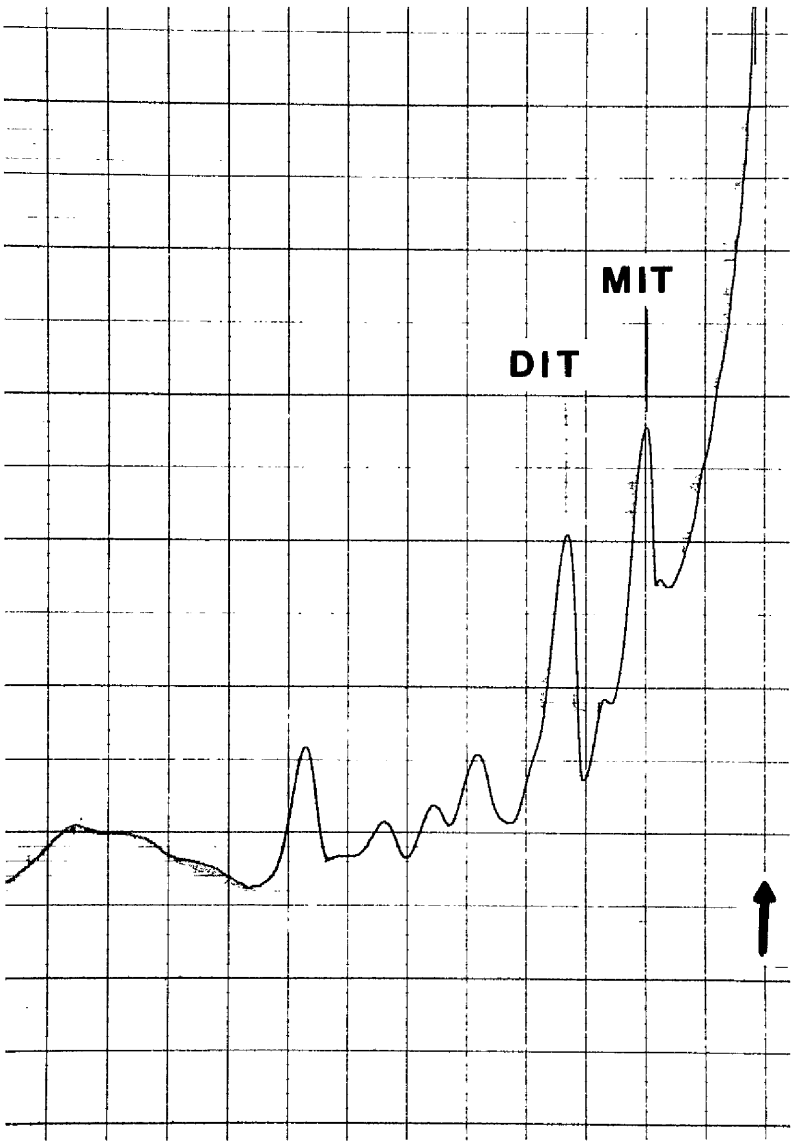


Figure 29 : Tracing of urine extract from patient S.S. (patient 57).

FINAL REMARKS

The chemistry of thyroid hormones began with the extraction of thyroxine by Kendall (1919) its isolation by Harington (1926) and the demonstration of triiodothyronine in blood by Gross & Pitt-Rivers in 1952. Yet reliable assays of these in blood and urine are a recent development, starting with competitive protein-binding by Ekins (1960) for thyroxine, followed by the emergence of useful, though cumbersome, methods for triiodothyronine (Naumann et al., 1967). At present, interest centres on the application of radioimmunoassay methods for the measurement of triiodothyronine and thyroxine in blood and urine.

There is a general agreement that both thyroxine and triiodothyronine are reversibly bound to a number of proteins, of which the best known are thyroxine-binding globulin, which has a high affinity for both hormones (especially thyroxine), albumin and for thyroxine particularly, thyroxine-binding peralbumin. In addition a large number of other proteins can be shown to interact with both triiodothyronine and thyroxine. Thus the protein binding of both hormones is exceedingly complex (Sterling, 1964). It is widely believed that the free or non-protein-bound thyroxine and triiodothyronine exert biological activity in serum while it is presumed that the protein-bound moities are biologically inactive as long as they remain bound. Thus direct measurement of circulating free thyroid hormones provide a better index of thyroid function.

The accepted methods used to measure the free hormone (based

on equilibrium dialysis, ultrafiltration or reverse flow electrophoresis) are complicated by artefacts due to the technical problems of separating the free from the far greater quantity of protein-bound hormone. Alternatively the indirect determination of the free thyroxine fraction, derived from measurement of the total serum concentration and simultaneous estimation of the vacant binding-sites for thyroxine on the thyroxine-binding globulin in the sample, while giving the "Free Thyroxine Index" falls short in that it only is of real value if there are abnormalities of the binding protein (Clark & Horn, 1965). The principle has not yet been routinely applied to free triiodothyronine.

Little is known of the renal excretion of thyroid hormones. As early as 1936 Mitolo (1936) demonstrated a small amount of free thyroxine in the urine of normal subjects. Later work, utilising radioactive iodine confirmed that organic iodine compounds do exist in urine, although in small quantities. Thus Joliot et al., (1944) found a small quantity of radioactive thyroxine and much larger amounts of radioactive diiodotyrosine in the urine of rabbits which had been given radioactive iodide. Roche et al., (1954) by injecting radioactive hormones in rats detected by chromatographic methods thyroxine and triiodothyronine in urine.

Bearing in mind that of the total thyroid hormones in the plasma, some will leak into the urine and particularly a proportion of the free hormones, estimation of these now being practical should add something to our understanding of the role of the free hormones. This has attracted interest because these free urinary hormone levels may reflect serum levels of the serum free hormones both in normal

and in various disease states. The main problem in the past has been the small but significant amounts of protein occurring in normal urine. This protein has led to nonspecific binding especially to interfere with radioimmunoassay and competitive protein binding methods of assay.

Estimation of urine and perhaps also serum free thyroid hormones by gas liquid chromatography has distinct possibilities.

Already both flame ionisation detection (FID) and electron capture detection (ECD) (^{63}Ni) have been used successfully in the analysis of derivatives of thyroid related iodoaminoacids both as standards and in serum (Stouffer et al., 1966; Jaakonmaki & Stouffer, 1967; Richards & Mason, 1966; Alexander & Scheig, 1968; Shahrokhi & Gehrke, 1968; Funakoshi & Cahnmann, 1969). While these authors have confirmed the practical nature of this form of quantitation, I have been unable to find published work on the application of the gas liquid chromatography methodology for the quantitation of free thyroid hormones in the urine.

Gas liquid chromatography cannot be used directly on crude urine as the urine contains a wide range of potentially volatile interfering compounds. An isolation step (or steps) therefore is unavoidable. The extraction procedure must permit the removal of protein and other substances which might interfere with the subsequent derivatisation and gas liquid chromatographic separation and detection of the hormones by electron capture detector. The use of electron capture detector dictates stringent requirements for the extraction steps. These are:

Firstly the small but significant quantities of urine protein

must be removed in the hope that all the protein-bound thyroid hormones will be removed leaving only the free fraction. This was achieved by washing the Dowex 50 column after the urine had been applied, with ammonium acetate (pH 8.5) (Sterling et al., (1969). Incidentally this wash also removed the urinary lipids and most of the aminoacids which included some but not all of the monoiodotyrosine and diiodotyrosine.

Secondly despite this column wash some aminoacids did appear in the ~~definite~~ definitive column extract. These were removed by the first thin layer chromatography development. No drastic step thereafter was used since there was a risk thereby of degrading the compounds sought.

Thirdly because of the notorious habit of the thyroid hormones to adhere to glassware surfaces, to avoid loss in this way the glassware was silanized with dimethyldichlorosilane, laborious as this was.

Fourthly pure extracts of thyroid hormones are relatively unstable to heat in their ionised form and display a measurable volatility at temperatures at which they decompose. This resistance to volatility is due to the fact that thyroid hormones have large molecules and the molecules are mutually associated through polar groups (amino, hydroxy and carboxyl). Also these hormones are considerably labile and often decompose on contact with the reactive surfaces of a chromatographic support or with metals. Thus it is necessary to prepare suitable derivatives more volatile, less polar and suitable for gas liquid chromatographic separation. Marked enhancement of volatility and suppression of the above undesirable

effects can be achieved by effecting a derivatisation that blocks the possibility of intermolecular association and reduces the reactivity of the compounds.

I have found that the pivalyl methyl esters are however the ideal derivatives of the thyroid hormones for gas liquid chromatography because they are not readily broken down either by traces of moisture or by the column oven temperature. Indeed they are less volatile than other derivatives and therefore they volatilise at higher temperatures. However the prepared esters require further purification before injection because the reaction reagents (pivalic anhydride and triethylamine) and the so formed pivalic acid have a high boiling point and therefore without purification contamination of the column and interference with quantitation would have resulted. This might be regarded as a drawback as it is not shared with other derivatives. The purification step was that described by Stouffer et al. (1966) being a simple thin layer chromatography system.

Fifthly only a few liquid phases fulfil the stability requirements for high temperature analysis of the dipivalyl esters of thyroid hormones. These temperatures range from 250 - 300°C. The linear polyorgano-siloxane OV-17 (methylphenyl organic groups) used by Hamilton (1973) possesses high chemical and thermal stabilities up to 350°C and I used this in my investigation. However, the 2 per cent coating employed by the above author was replaced by 3 per cent and the packed column was shortened from 5 to 3 foot for better baseline separation.

Finally on the problems of methodology, despite the various purification steps introduced, a large number of peaks were obtained from test samples of urine. These peaks clearly represented derivatives of other aminoacids but fortunately it was found possible to cause these to run ahead of the thyroid hormone derivatives by using a temperature programmed procedure rather than isothermal conditions. Among these peaks can be identified monoiodotyrosine, diiodotyrosine and diiodothyronine, and therefore this finding might facilitate future studies designed to elucidate thyroid hormone metabolism. It may be that the coupling of gas chromatography to mass spectrometry would add to the usefulness of the method especially if the investigator was interested in the many unknown peaks which I caused to run ahead by the temperature programming.

In conclusion, the analysis of the free thyroid hormones in urine presents a major analytical challenge. Not only is the urine itself extremely complex, but the free hormones are present along with a variable amount of metabolites most of which are not identifiable. Further, the large molecular weight of these thyroid hormones (approximately 1,000), the lability of their iodine atoms, their tendency to adsorb on solid surfaces and their presence and availability in only trace amounts in urine (in the free form), were the problems which had to be overcome in the present work.

The results which I have obtained, while perhaps few, have given me an opportunity to evaluate the overall reliability of a gas liquid chromatography method and interestingly there was no great disparity between my results and those of free urinary thyroid

hormone levels reported by other authors using steady-state gel filtration (Burke & Shakespear, 1976).

When I applied reasonable logic to the interpreting of my data and those of these authors, my finding that the tubular reabsorption value for both thyroxine and triiodothyronine were in the range expected for small molecular weight compounds was gratifying. This gives credence to the gas liquid chromatography method of which this thesis is the testimony.

Using the developed methodology, the levels of the urinary thyroid hormones in euthyroid and hypothyroid children were estimated. It is of interest that there is neither reported values for urinary free thyroid hormones as determined by gas liquid chromatography nor are there values for children by any other method. However when my values were compared with those reported for adults (by competitive protein-binding and radioimmunoassay) my values were one-third of those values. These data suggest either that the current methods overestimate the urinary thyroid hormones by the inclusion of additional material or my values are low because of loss of hormone during the analytical procedure. The latter suggestion can be ruled out since a correction factor is always available in the tracer ^{14}C-thyroxine. Another possibility to account for the disparity is that the level of urinary thyroid hormones in children is lower than that of adults. The low levels in neonates with a steady increase during childhood might suggest this. However such a large difference as one-third was not expected since comparable values for the serum free thyroxine in both children and adults does not differ by such a magnitude.

On the other hand my urinary values are not significantly

different from the values of free urinary thyroid hormones in adults as determined after steady-state gel filtration (Burke & Shakespear, 1976). In support of the above is the calculated clearance values of both triiodothyronine and thyroxine assuming normal renal function. These were in the range expected for small molecular weight compounds. Also there is the clear discrimination between the values found in the euthyroid and in the hypothyroid states by my method.

Thus although tedious the gas liquid chromatography methodology appears to offer more specificity and less interference than methods previously reported.

REFERENCES

Albright, E.C., Larson, F.C. and Tust, R.H. (1954).
In vitro conversion of thyroxine to triiodothyronine by kidney slices.
Proc. Soc. Exp. Biol. Med., 86 : 137.

Albright, E.C., Tomita, K. and Larson, F.C. (1959).
In vitro metabolism of triiodothyronine.
Endocrinology, 64 : 208.

Albucasis (10th century)
cited by Lager, P. in "History of goitre".
Endemic Goitre, World Health Organisation Monograph Series No. 44 (1960). Geneva.

Alexander, N.M. and Sheig, R. (1968).
Gas chromatography of the trimethylsilyl derivatives of iodotyrosines and iodothyronines.
Anal. Biochem., 22 : 187.

Andersen, J.H. (1975).
In Endocrine and genetic diseases of childhood and adolescence. (ed. L.I. Gardener), p. 240.
London : Saunders Co.

Backer, E.T., Postmes, T.J. and Wiener, J.D. (1967).
Determination of thyroid hormones in serum by means of cation exchange resin and chloric acid digestion.
Clin. Chim. Acta, 15 : 77.

Baumann, E. (1895).
Uber das normale Vorkommen Von Jod im Thierkörper.
Phys. Chem., 21 : 319.

Beisel, W.R., Cos, J.J., Horton, R., Chao, P.Y. and Forsham, P.H.(1964).
Physiology of urinary cortisol excretion.
J. Clin. Endocr. Metab., 24 : 887.

Black, E.G., Griffiths, R.S., Hoffenberg, R., Leatherdale, B. (1973).
Measurement of urinary thyroxine.
Lancet, 1 : 152.

Black, E.G., Griffiths, R.S., Finucane, J. and Hoffenberg, R. (1975).
Radioimmunoassay of triiodothyronine and thyroxine in serum and urine.
In Thyroid Hormone Metabolism.
Proceedings of International Thyroid Hormone Conference, Glasgow, 1974.
(Eds. W.A. Harland and J.S. Orr), p. 347.
Academic Press.

Bowers, C.Y., Schally, A.V., Kastin, A., Arimura, A., Schalch, D.S. Gual, C., Gastineda, E. and Folkers, K. (1971). Synthetic thyrotrophin-releasing hormone; Activity in men and women, specificity of action, inhibition by triiodothyronine, and activity orally. J. Med. Chem., 14 : 477.

Braverman, L.E., Ingbar, S.H. and Sterling, K. (1970). Conversion of thyroxine to triiodothyronine in athyreotic human subjects. J. Clin. Invest., 49 : 855.

Burke, C.W. (1974). Hormones in urine : Uses and misuses. J. Roy. Coll. Physcns. (Lond.), 8 : 335.

Burke, C.W. and Beardwell, C.G. (1973). Cushing's syndrome : An evaluation of the clinical usefulness of urinary free cortisol and other urinary steroid measurements in diagnosis. Q.J. Med., 42 : 175.

Burke, C.W. and Shakespear, R.A. (1975). Renal excretion of triiodothyronine and thyroxine and diagnostic value of urinary triiodothyronine and thyroxine assays. In Thyroid Hormone Metabolism. Proceedings of International Thyroid Hormone Conference, Glasgow, 1974. (Eds. W.A. Harland and J.S. Orr), p 319. Academic Press.

Burke, C.W. and Shakespear, R.A. (1976). Triiodothyronine and thyroxine in urine. II. Renal handling and effect of urinary protein. J. Clin. Endocr. Metab., 42 : 504.

Burke, C.W., Shakespear, R.A. and Fraser, T.R. (1972). Measurement of thyroxine and triiodothyronine in human urine. The Lancet, 2 : 1177.

Burke, C.W., Fraser, T.R., Graves, L., Shakespear, R.A. (1973). The urinary excretion of triiodothyronine and thyroxine in man. J. Physiol. (Lond.), 231 (2) : 62P.

Cavalieri, R.R., Moser, C., Martin, P., Shamos, D. and Perez-Mendez, V. (1975). Kinetics of renal uptake of L-triiodothyronine in man. In Thyroid Hormone Metabolism. Proceedings of International Thyroid Hormone Conference, Glasgow, 1974. (Eds. W.A. Harland and J.S. Orr), p. 307. Academic Press.

Chan, V. (1974).
The assay of urinary thyroid hormones for assessing thyroid function.
Ann. Clin. Biochem., 11 : 120.

Chan, V. and Landon, J. (1972).
Urinary thyroxine excretion as index of thyroid function.
Lancet, 1 : 4.

Chan, V., Besser, G.M., Landon, J. and Ekins, R.P. (1972).
Urinary triiodothyronine as index of thyroid function.
Lancet, 2 : 253.

Chan, V., Smith, M., Hale, P.J. and Landon, J. (1973).
Thyroid function in the neonate.
Clin. Endocr., 2 : 333.

Clark, F. and Horn, D.B. (1965).
Assessment of thyroid function by the combined use of the serum protein bound iodine and resin uptake of ^{125}I-triiodothyronine.
J. Clin. Endocr., 25 : 39.

Cooper, A. (1827).
Observations on the thyroid gland by T.W. King with "Notes on the structure of the thyroid gland" by Sir Astley Cooper.
Guy's Hosp. Rep., 1 : 448.

DeLange, F.M. and Ermans, A.M. (1967).
Le metabolism de l'iode a la puberte.
Rev. France Etudes Clin. Biol., 12 : 815.

Docter, R. and Hennemann, G. (1971).
Estimation of thyroid hormones by gas liquid chromatography.
Clin. Chim. Acta, 34 : 297.

Ekins, R.P. (1960).
The estimation of thyroxine in human plasma by an electrophoretic technique.
Clin. Chim. Acta, 5 : 453.

Ellis, S.M. and Ekins, R.P. (1973).
The radioimmunoassay of free (diffusable) T_3 and T_4 concentrations in serum.
J. Endocr., 59 : XLIII.

Etting, E.C. and Barker, S.B. (1959).
Metabolism of thyroxine during prolonged kidney cortex incubation.
Endocrinology, 64 : 753.

Finucane, J.F. (1976).
Random urine tests in the assessment of thyroid function.
Ir. J. Med. Sci., 145 : 195.

Finucane, J.F., Griffiths, R.S., Black, E.G. and Hall, C.L. (1977).
Effects of chronic renal disease on thyroid hormone metabolism.
Acta Endocrin. 84 : 750.

Fisher, D.A. (1971).
In The Thyroid (Ed. S.C. Werner and S.H. Ingbar), pp. 292 - 296.
New York : Harper & Row.

Floyd, J.C. Jr., Beierwaltes, W.H., Dodson, V.N. and Carr, E.A. (1960).
Defective iodination of tyrosine a cause of nodular goitre?
J. Clin. Endocr., 20 : 881.

Folk, E.V., Bollman, J.L. and Grindlay, J.H. (1960).
Conjugates of triiodothyronine and its metabolites.
Endocrinology, 67 : 419.

Freinkel, N. and Ingbar, S.H. (1955).
The relationship between metabolic activity and iodide concentrating capacity of surviving thyroid slices.
J. Clin. Endocr., 15 : 442.

Funakoshi, K. and Cahnmann, H.J. (1969).
Gas chromatography determination of iodinated compounds.
Anal. Biocham., 27 : 150.

Gaitan, J.E., Wahner, H.W., Gorman, C.A. and Jiang, N.S. (1975).
Measurement of triiodothyronine in unextracted urine.
J. Lab. Clin. Med., 86 : 538.

Goldsmith, R.E., Rauth, J.L., Kloth, R. and Dahlgren, J. (1967).
Observations on the relationship between the maximal thyroxine binding capacities of thyroxine binding interalpha globulin and thyroxine binding concentration and sexual maturity in adolescence.
Acta Endocr., 54 : 494.

Goldstein-Golaire, J. and DeLange, F. (1971).
Serum thyrotrophin level during growth in man.
Europ. J. Clin. Invest., 1 : 405.

Gordon, A.H. and Eastoe, J.E. (1964).
Practical chromatographic techniques, p.131.
London : George Newnes Ltd.

Gray, C.E. (1973).
The possible significance of dehydroepiandrosterone in cystic fibrosis.
Ph. D. Thesis, Glasgow University.

Greer, M.A. and Smith, G.E. (1954).
Method for increasing the accuracy of the radioiodine uptake as a test of thyroid function by the use of dessicated thyroid.
J. Clin. Endocr. Metab., 14 : 1374.

Gross, J. and Pitt-Rivers, R. (1952).
The identification of 3:5:3'-L-triiodothyronine in human plasma.
Lancet, 1 : 439.

Hamilton, W. (1973).
G.L.C. quantitation of thyroid hormones.
Acta Paediat. Scand., 62 : 94.

Harrington, C.R. (1926).
Chemistry of thyroxine. I. Isolation of thyroxine from the thyroid gland.
Biochem. J., 20 : 293.

Harrington, C.R. (1944).
Newer knowledge of the biochemistry of thyroid gland.
J. Chem. Soc., 1 : 193.

Harrington, C.R. and Barger, G. (1927).
Chemistry of thyroxine. III. Constitution and synthesis of thyroxine.
Biochem. J., 21 : 169.

Hoffenberg, R. (1973).
Triiodothyronine.
Clin. Endocr., 2 : 75.

Hoffenberg, R., Lauson, A.M., Ramsden, D.B. and Raw, P.J. (1974).
The application of mass spectrometry to the study of thyroxine and related compounds in biological fluids.
In Mass Spectrometry in Biochemistry and Medicine.
(Eds. A. Frigerio and N. Castagnoli), p.303.
New York : Raven Press.

Horning, E.C. (1968).
In Gas Phase Chromatography of Steroids.
(Eds. K.B. Nik-Nes and E.C. Horning), p. 12.
Berlin : Springer-Verlag.

Howitz, F. (1893).
The treatment of myxoedema by feeding with thyroid glands.
Brit. Med. J., 1 : 266.

Hufner, M. and Hesch, R.D. (1973).
Triiodothyronine determinations in urine.
Lancet, 1 : 101.

Hung, W. (1975).
Physiological changes in the thyroid during childhood and adolescence.
In Endocrine and Genetic Disease of Childhood and Adolescence.
(Ed. L.I. Gardener), 2nd. ed.,
London : W.B. Saunders Co.

Husek, P. (1974).
Gas chromatographic behaviour of amino acid oxazolidinones : Response to flame ionization and electron capture detectors.
J. Chromat., 91 : 483.

Husek, P. and Macek, K. (1975).
Gas chromatography of amino acids.
J. Chromat., 113 : 139.

Hutchinson, J. (1969).
The thyroid gland.
In Paediatric Endocrinology (Ed. D. Hubble).
Oxford : Blackwell Scientific.

Inczedy, J. (1966).
In Analytical Applications of Ion Exchngers.
(Ed. I. Buzas), p.127.
London : Pergamon Press.

Jaakonmaki, P.I. and Stouffer, J.E. (1967).
Gas chromatographic analysis with electron capture detection of thyroid hormones.
J. Chromat., 5 : 303.

Jantsch, M. (1948).
Der Kropf und seine Behandlung. Eine geschichtliche Ubersicht, Wien.
Cited by Langer, P. (1960) in Endemic Goitre.
World Health Organisation, monograph series no. 44, Geneva.

Joliot, F., Courrier, R., Horeau, A. and Sue, P. (1944).
Chimie Physique Biologigue - Sur l'obtention de la thyroxine marquee par la radioiode et son comportement sans l'organisme.
Comp. rend Acad. d. Sci., 218 : 769.
Cited by Rall, J.E. (1950).

Katz, A.I., Emmanouel, D.S. and Lindheimer, M.D. (1975).
Thyroid hormone and the kidney.
Nephron, 15 : 223.

Kay, C., Abrahams, S. and McLain, P. (1966).
The weight of normal thyroid glands in children.
Arch. Path., 82 : 349.

Kendall, E.C. (1919).
Isolation of the iodine compound which occurs in the thyroid.
J. Biol.Chem., 39 : 125.

King, T.W. (1836).
Observations on the thyroid gland.
Guy's Hosp. Rep., 1 : 429.

Kocher, T. (1883).
Uber Kropfextripation Und ikre Folgen.
Arch. Klin. Chim., 29 : 254.

Lainlaw, J.C. (1949).
Nature of the circulating thyroid hormone.
Nature (London), 164 : 927.

Lamburg, B.A., Kantero, R.L., Saarinen, P. and Widholm, O. (1973)a.
Endocrine changes before and after the menarche.
IV. Serum thyrotrophin in female adolescents.
Acta Endocrin.,74 : 695.

Lamburg, B.A., Kantero, R.L., Saarinen, P. and Wildholm, O. (1973)b.
Endocrine changes before and after the menarche. III. Total thyroxine and "free thyroxine index" and the binding capacity of thyroxine-binding proteins in female adolescents.
Acta. Endocrin.,74 : 685.

Langer, P. (1960).
History of goitre.
In Endemic Goitre.
World Health Organisation, monograph series No. 44, p. 18., Geneva.

Larson, F.C. and Albright, E.C. (1965).
The specificity of thyroxine binding by serum alpha globulin.
Endocrinology, 56 : 737.

Lee, N.D. and Pileggi, V.J. (1971).
Measurement of free thyroxine in serum.
Clin. Chem., 17 : 166.

Lemarchand-Beraud, T., Genazzani, A.R., Bagnoli, F., Casoli, M. (1972).
Thyroid function in the premature and full term newborn.
Acta Endocrin., 70 : 445.

Liek, E. (1929).
Das Kropfrätsel, München.
Cited by Langer, P. (1960) in Endemic Goitre.
World Health Organisation, monograph series no.44, Geneva.

Malvaux, P., Beckers, C. and De Visscher, M. (1965).
Dynamic studies on the inorganic iodine compartment and its exchanges during adolescence.
J. Clin. Endocr. Metab., 25 : 817.

Malvaux, P., De Nayer, P., Beckers, C., Van Den Schrieck, H.G. and De Visscher, M. (1966).
Serum free thyroxine and thyroxine binding proteins in male adolescents.
J. Clin. Endocr. Metab., 26 : 459.

Marks, J.F., Hamlin, M. and Zack, P. (1966).
Neonatal thyroid function. II. Free thyroxine in infancy.
J. Paediatr., 68 : 559.

Matthews, J.S., Pereda, A.L. and Aguilera, A. (1962).
The quantitative analysis of steriods by thin layer chromatography.
J. Chromat., 9 : 331.

Merck Index (1968).
(Ed. Stecher, P.G.).
Merck & Co., U.S.A.

Mettler, C.C. (1947).
In History of Medicine, Toronto.
Cited by Langer, P. (1960) in Endemic Goitre.
World Health Organisation, monograph series no.44, Geneva.

Mitolo, M. (1936).
Tirosinuria umana normale individuale.
Endocrin. e. Patol. Costituz., 11 : 197.
Cited by Rall, J.E. (1950).

Mosier, H.D. (1975).
Interpretation of thyroid function tests.
In "Endocrine and genetic diseases of childhood and adolescence".
Edited by Gardner, L.I. (2nd. edition).
London : W.B. Saunders Co.

Murray, G.R. (1891).
Note on the treatment of myxoedema by hypodermic injections of an extract of the thyroid gland of a sheep.
Brit. Med. J., 2 : 796.

Nauman, J.A., Nauman, A. and Werner, S.C. (1967).
Total and free triiodothyronine in human serum.
J. Clin. Invest., 4 : 1346.

Nihei, N., Gershengorn, M.C., Mitsuma, T., Stringham, L.R., Cordy, A., Kuchmy, B. and Hollander, C.S. (1971).
Measurements of triiodothyronine and thyroxine in human serum by gas liquid chromatography.
Anal. Biochem., 43 : 433.

Oddie, T.H. and Fisher, D.A. (1967).
Protein bound iodine level during childhood and adolescence.
J. Clin. Endocr. Metab., 27 : 89.

Ogawara, H., Bilstad, J.M. and Cahnmann, H.J. (1972).
Iodoaminoacid distribution in thyroglobulin iodinated in vivo and in vitro.
Biochim. Biophys. Acta, 257 : 339.

Oswald, A. (1899).
Die Eiweisskörper der Schilddüse.
Ztschr. F. Physiol. Chem., 27 : 14.

Pastrana, S.L., Beltran, M.A., Gonzalez, P.J., Cuevas, L.M., Maisterrena, J.A. and Valverde-R, C. (1974).
Urinary thyroxine assay.
Rev. Invest. Clin. (Mex.), 26 : 133.

Pastrana, S.L., Gonzalez, J.P., Maisterrena, J.A., Valverde-R, C.(1975).
Clinical value of urinary free thyroxine assays.
Rev. Invest. Clin. (Mex.), 27 : 179.

Petersen, B.A., Hanson, R.N., Giese, R.W. and Karger, B.L. (1976).
Picogram analysis of free triiodothyronine and free thyroxine hormones in serum by equilibrium dialysis and electron capture gas chromatography.
J. Chromat., 126 : 503.

Pattison, J.B. (1969).
A programmed introduction to gas liquid chromatography. p.216.
London : Heyden & Son Ltd.

Pittman, C.S., Chambers, J.B. and Read, V.H. (1971).
The extrathyroidal conversion rate of thyroxine to triiodothyronine in normal man.
J. Clin. Invest., 50 : 1187.

Pittman, C.S., Buck, M.W. and Chambers, J.B. (1972).
Urinary metabolites of ^{14}C-labelled thyroxine in man.
J. Clin. Invest., 51 : 1759.

Pitt-Rivers, R. and Schwartz, H.L. (1967).
Iodoaminoacids and related compounds.
In Chromatographic and Electrophoretic Techniques.
(Ed. Ivor Smith), 3rd. ed., Vol.1, p. 228.
London : Interscience Publishers.

Plaskett, L.G. (1962).
Catabolism of doubly labelled thyroxine in vivo.
Nature, 195 : 961.

Pochin, E.E. (1950).
Investigation of thyroid function and disease
with radioactive iodine.
Lancet, 2 : 41.

Rahill, W.J. (1969).
Renal function in infancy;
in Textbook of Paediatrics.
(Eds. W.E. Nelson, V.C. Vaughan and R.J. McKay), 9th. ed.,
London : Saunders Co.

Rall, J.E. (1950).
Iodine compounds in the blood and urine of man.
J. Clin. Endocr., 10 : 996.

Rasmussen, H. (1956).
Thyroxine metabolism in nephrotic syndrome.
J. Clin. Invest., 35 : 792.

Rastogi, G.K., Sawhney, R.C., Sinha, M.K., Thomas, Z. and Devi, P.K.
(1974).
Serum and urinary levels of thyroid hormones
in normal pregnancy.
Obstet. Gynaec., 44 : 176.

Rauth, J.L., Knox, M.D. and Goldsmith, R. (1964).
Effect of sexual maturity on the serum concentration
of "hormonal iodine" in adolescence.
J. Paediatr., 64 : 697.

Reverdin, J.L. and Reverdin, A. (1883).
Note sur vingt-deux operations de goitre.
Rev. Med. Suisse Rom., 3 : 169.

Richards, A.H. and Mason, W.B. (1966).
Gas chromatographic separation of some iodine
compounds of serum.
Anal. Chem., 38 : 1751.

Robbins, J. and Rall, J.E. (1955).
Effect of triiodothyronine and other thyroxine
analogues on thyroxine-binding in human serum.
J. Clin. Invest., 34 : 1331.

Robbins, J. and Rall, J.E. (1960).
Proteins associated with the thyroid hormones.
Physiol. Rev., 40 : 415.

Roche, J. and Michel, R. (1955).
Nature, biosynthesis and metabolism of thyroid hormones.
Physiol. Rev., 35 : 583.

Roche, J., Lissitzky, S. and Michel, R. (1952)a.
Sur le metabolism de la triiodothyronine et sur sa
biosynthese en tant qu'hormone triiodothyronine.
CR. Soc. Biol. (Paris), 146 : 1474.

Roche, J., Michel, R. and Tata, J. (1952)b.
Sur le metabolism de la thyroxine marquee par l'iode
radicactif en differentes positions.
CR Soc. Biol. (Paris), 146 : 1003.

Roche, J., Michel, R. and Tata, J. (1954).
Sur la nature des combinaisions iodes excretees par
le foie et le rein apres administration de L-thyroxine
et de L-3,5,3'-triiodothyronine.
Biochim. Biophys. Acta, 5 : 500.

Rolland, M., Aquaron, R. and Lissitzky, S. (1970).
Thyroglobulin iodoamino acids estimation after digestion
with pronase and leucylaminopeptidase.
Anal. Biochem., 33 : 307.

Rolleston, H.D. (1936).
The endocrine glands in health and disease
with a historical review.
London : Oxford University Press.

Rosenberg, I.N. (1951).
The nature of the circulating thyroid hormone
in Grave's disease.
J. Clin. Invest., 30 : 1.

Ruskin, T.W.Av., Tang, S.C., Shenkman, L., Mitsuma, T.
and Hollander, C.S. (1973).
Serum triiodothyronine concentration in infancy,
childhood, adolescence and paediatric thyroid disorders.
J. Clin. Endocr. Metab., 37 : 235.

Sayed, Ismail Al-Jurjani (circa AD 1136).
Cited by Langer, P. (1960). Endemic Goitre.
World Health Organisation, monograph series no.44, Geneva.

Schachner, H., Franklin, A.L. and Chaikoff, I.L. (1944).
On the in vitro accumulation of inorganic iodide by surviving
thyroid tissue with radioactive iodine as indicator.
Endocrinology, 34 : 159.

Shahrokhi, F. and Gehrke, C.W. (1968).
Quantitative gas liquid chromatography of iodine-containing amino acids.
Anal. Biochem., 24 : 281.

Shakespear, R.A. and Burke, C.W. (1976).
Triiodothyronine and thyroxine in urine.
I. Measurement and application.
J. Clin. Endocr. Metab., 42 : 494.

Stokelenburg, G.J. van, Stoop, J.W. and Stam, P.J. van der (1970).
Normal values of serum protein bound iodine in healthy children aged between 4 and 13 years; biochemical evidence for the existence of the juvenile or mid-growth spurt.
Acta Endocrin., 65 : 477.

Sterling, K. (1964).
Thyroxine in blood.
Mayo Clin. Proc., 39 : 586.

Sterling, K. (1971).
Free thyroxine and triiodothyronine.
CRC Crit. Rev. Clin. Lab. Sci., 2 : 223.

Sterling, K. and Hegedus, A. (1962).
Measurement of free thyroxine concentration in human serum.
J. Clin. Invest., 41 : 1031.

Sterling, K., Bellabarba, D., Newman, E.S. and Brenner, M.A. (1969).
Determination of triiodothyronine concentration in human serum.
J. Clin. Invest., 48 : 1150.

Sterling, K. and Lazarus, H. (1977).
The thyroid and its control.
Ann. Rev. Physiol., 39 : 349.

Sorimachi, K. and Ui, N. (1975).
Ion-exchange chromatographic analysis of iodothyronines.
Anal. Biochem., 67 : 157.

Stouffer, J.E., Jaakonmaki, P.I. and Wenger, T.J. (1966).
Gas liquid chromatographic separation of thyroid hormones.
Biochim. Biophys. Acta, 127 : 261.

Street, H.V. (1969).
The use of gas-liquid chromatography in clinical chemistry.
Adv. Clin. Chem., 12 : 236.

Tabachnick, M., Has, Y. and Korcek, L. (1971).
Effect of peptide derivatives of thyroxine on the binding of ^{125}-I-Thyroxine to purified human thyroxine-binding globulin.
Endocrinology, 89 : 606.

Tajuddin, M. and Elfbaum, S.G. (1973).
Evaluation of "Dexil 300 GC" as stationary phase in gas chromatography of triiodothyronine and thyroxine.
Clin. Chem., 19 : 109.

Tanner, J.M. (1962).
Growth at adolescence. 2nd. edition.
Oxford : Blackwell Scientific Publications.

Volpert, E.M., Grinberg, R. and Werner, S.C. (1963).
In vivo deiodination of labelled-L-thyroxine to 1-3,5-3'-triiodothyronine in mouse and human pituitaries.
J. Clin. Endocr., 23 : 140.

Volpert, E.M., Kundu, N. and Dawidzik, J.B. (1970).
N,O-dipivalyl methyl esters of the thyroid hormones and related compounds; synthesis, purification and gas chromatography.
J. Chromat., 50 : 507.

Werner, S.C. and Spooner, M. (1955).
New and simple test for hyperthyroidism employing L-triiodothyronine and 24-hour I-131 uptake method.
Bull. NY Acad. Med., 31 : 137.

West, C.D., Wayne, A.W. and Chavre, V.J. (1965).
Thin-layer chromatography for thyroid hormones.
Anal. Biochem., 12 : 41.

Wilkinson, J.W. and Bowden, C.H. (1960).
In Chromatographic and Electrophoretic Techniques.
(Ed. Ivor Smith), 2nd ed., Vol.1, p. 167.
London : Interscience Publishers.

Urinary free thyroid hormones in children:

An assay by gas liquid chromatography

by

Abdelhamid Abdelkadir Mansour

M.B.B.S., B.Ch.D., D.C.H.

A SUMMARY

of the thesis submitted to the
University of Glasgow
for the degree of Doctor of Philosophy

April 1978

Due to the trace levels of the free thyroid hormones in urine a variety of methods based on radiochemical techniques have been used for estimating free triiodothyronine and thyroxine, viz. competitive protein binding and radioimmunoassay. While these techniques are currently employed, they suffer certain disadvantages, among which are the cross reaction of the urinary thyroid hormone metabolites with antibody in the radioimmunoassay system (Gaitan et al., 1975) and the competing of these metabolites for binding sites in the competitive-binding system (Finucane, 1976). Additionally, the above methods do not discriminate between bound and free urinary thyroid hormones (Burke & Shakespear, 1976).

In this work the use of gas liquid chromatography as a method for the quantitation of urinary thyroid hormones is reported for the first time.

Following the addition of thyroxine-2-^{14}C for recovery purposes, aliquots of urine (20ml.) were made acidic by the addition of 5M hydrochloric acid and the thyroid hormones extracted with cation exchange (Dowex 50W-X2(H^+)) column chromatography (Sterling et al., 1969). Purification of the extracted residues was effected by thin layer chromatography (Chan & Landon, 1972). The dried residues were derivatised by a two-steps method

(Hamilton, 1973). First the carboxyl group was methylated with dry methanol in the presence of hydrogen chloride gas for 30 minutes at 70°C, to form the methyl ester. The latter was dried under nitrogen and then acylated with trimethyl acetic anhydride (pivalic anhydride) on the amino nitrogen and phenolic hydroxyl groups in the presence of triethylamine for 30 minutes at 110°C to form the methyl N,O-dipivalyl derivative. Following subsequent purification by thin layer chromatography (Stouffer et al., 1966), quantitation of N,O-dipivalyl methyl esters of triiodothyronine and thyroxine was made by electron capture detection and programmed thermal oven conditions on a Pye 104 gas liquid chromatograph. A correction was made for the recovery rate of radioactively labelled thyroxine added initially to the urine samples.

Using the developed methodology, the levels of the urinary thyroid hormones in 50 euthyroid and 7 hypothyroid children were estimated. It is of interest that there is neither reported values of the urinary thyroid hormones by gas liquid chromatography nor other method for children.

In the series of euthyroid children whose age ranged from 3 to 14 years the mean daily excretion rate of triiodothyronine was 348.66 $\pm$ 50.41ng. and 484.20 $\pm$ 85.15ng. for thyroxine. There was a small upward increase in the excretion rates for both triiodo-

thyronine and thyroxine with age and this correlates with the body requirements for constant free plasma levels of triiodothyronine and thyroxine. From the values found, the respective tubular reabsorption factors were calculated and both compounds behaved as for low molecular weight compounds. The tubular reabsorption of triiodothyronine was 90.02 per cent and the tubular reabsorption of thyroxine was 94.03 per cent.

The reported method here while prolix has the advantage over other current methods, estimates the true free level of urinary thyroid hormones and also in that it discriminates between the levels found in euthyroid and hypothyroid patients. The urinary levels found in the hypothyroid patients taken with the respective plasma levels indicated that there was a specific plasma level below which neither triiodothyronine nor thyroxine appeared in the urine. Additionally, the reported method is a step forward to the use of gas liquid chromatography-mass spectrometry combination. By this latter, a unique in its power to identify and quantitate at femtomole levels unknown compounds would rapidly increase our understanding of thyroid metabolism and excretion.